EXPLORING CAUSATIVE AND MODIFYING FACTORS OF METAL MINE EFFLUENT TOXICITY USING SHORT-TERM MULTI-TROPHIC ARTIFICIAL STREAM SYSTEMS

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By

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Abstract

Metal mines release treated effluents that contain a variety of metals, metalloids, and organics into the aquatic environment. A number of metal mine effluents (MMEs) have been found to contribute to adverse effects in fish and benthic invertebrates, such as decreased diversity and density, however the specific causal factors of toxic responses during chronic exposures to the MMEs are often unknown. Therefore, the overall objective of this dissertation was to explore causative and modifying factors of MME toxicity to a resident fish species, the fathead minnow (*Pimephales promelas*), during chronic, multi-trophic exposures. The representative MME used in this study was the process water effluent (PWE) of a Canadian metal mine, which is released into Junction Creek in Sudbury, Ontario, Canada. Chronic exposure to the MME has been a source of decreased reproductive output in fathead minnows in several previous studies, however, these same studies were not able to determine the potential causal factors of the reproductive impairment. In order to address the overall objective, several laboratory mesocosm studies were conducted, which consisted of three separate components. The first component included exploring several metals (Cu, Ni, and Se; alone and in mixture) that are consistently present in the MME and are known to cause toxicity at fairly low concentrations as potential causes for decreased egg production in fathead minnows. The second component included evaluating the role of decreased food availability (a possible indirect effect of MME in the receiving environment) as a potential cause of decreased egg production in fathead minnows. The third and final component included examining the role of water chemistry [(increased alkalinity and dissolved organic carbon (DOC)] as potential modifying factors of chronic MME toxicity to fathead minnows.

In general, my results suggest that the metals present in the MME likely do not contribute directly to decreased reproductive performance in fathead minnows during chronic exposures, under the conditions examined. Instead, the MME appears to decrease food availability, therefore indirectly influence fathead minnow egg production. Furthermore, water chemistry modifications tested in this thesis were not able to entirely mitigate the reproductive effects in fish induced by the MME, although they did improve egg production relative to unmodified MME. Metal concentrations in fish tissues were not influenced by increases to alkalinity or DOC level in the exposure water, suggesting that bioavailability of metals during chronic exposure to metal-mixtures cannot be fully explained based on our understanding of metal complexation with abiotic ligands (inorganic and organic) during single metal or acute exposures. From a regulatory perspective, water chemistry modifications may somewhat improve fathead minnow reproductive performance during chronic exposure to the MME, however the MME would still not be entirely free of effects relative to the uncontaminated water. Future studies should focus on understanding the factors responsible for decreased food availability in MME-impacted aquatic ecosystems, and further explore potential approaches for ameliorating effluent quality.

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Abbreviations and Acronyms

µg/L – microgram per litre

µS/cm – microsiemens per centimetre

ANOVA – analysis of variance

APHA – American Public Health Association

AREB – Animal Research Ethics Board

BLM – Biotic Ligand Model

CCME – Canadian Council of Ministers of the Environment

DO – dissolved oxygen

DOC – dissolved organic carbon

d.w. – dry weight

EEM – Environmental Effects Monitoring

EPA – Environmental Protection Agency

FHM – fathead minnow

GSI – gonadosomatic index

HDPE – high density polyethylene

ICP-MS – inductively coupled plasma-mass spectrometry

IOC – investigation of cause

K – condition factor

KS – Kolmogorov-Smirnov

LSI – liver somatic index

mg/L – milligram per litre

MM – metal mixture

MME – metal mine effluent

MMER – Metal Mining Effluent Regulations

MWE – mine water effluent

NOM – natural organic matter

PWE – process water effluent

PWE-HF – process water effluent, high food

PWE –LF – process water effluent, low food

RO – reverse osmosis

RW – reference water

RW-HF – reference water, high food

RW-LF – reference water, low food

SE – standard error

SWE – surface water effluent

TOC – total organic carbon

UCACS – University of Saskatchewan Committee on Animal Care and Supply

w.w. – wet weight

1 CHAPTER 1 - GENERAL INTRODUCTION

1.1 INTRODUCTION TO METAL MINE EFFLUENTS

Metal mines have the potential to alter landscapes and waterways by the physical removal of metals, earth, and rock, as well as through the waste resulting from these processes. Mining waste includes treated waste rock seepage and tailings from ore extraction in the form of effluents that are discharged into surface waters located near the mining operations (Dubé et al. 2005). Metals and other contaminants that remain in the effluents can be harmful to the environment, although the effluent characteristics that contribute to toxic effects are dependent on ore type, methods of mineral extraction, and hydrogeology (Clarke 1974, Kelly 1988, Lottermoser 2007). Metals, including those found in effluents, have the ability to negatively impact aquatic life when released into the environment due to their persistence, ability to bioaccumulate, and inherent toxicity (Atchison et al. 1987). Accordingly, metal mine operations perform complex treatment procedures on their effluents to ensure toxicity is eliminated or minimized in order to maintain water quality and prevent damage to the receiving aquatic ecosystem. Effluent treatment normally consists of precipitation of unwanted contaminants and adjustment of pH to match that in the receiving waters.

Although treatment improves effluent quality, problems can still remain. Effects in invertebrates, such as metal bioaccumulation (Kiffney and Clements 1993), decreased survival, abundance, growth, and hatch rates (Hruska and Dubé 2004, 2005), and decreased species richness (Hickey and Clements 1998) have been documented as a result of exposure to metal mine effluents (MMEs) or metal contaminated waters. Effects have also been observed in fish under similar types of exposures to MMEs or metal contaminated waters. For example, increased concentrations of metals in tissues (Driessnack et al. 2011, Dubé et al. 2005), behavioural changes (Gerhardt 1998, McPherson et al. 2004), increased larval deformities and

delayed development (Driessnack et al. 2011, Jezierska et al. 2009), decreased lipid storage and growth (Bennett and Janz 2007), as well as decreased fecundity (Driessnack et al. 2011, Franssen 2009, Rozon-Ramilo et al. 2011a, 2011b) have been reported in fish during exposure to MMEs or found in metal contaminated lakes and rivers. These types of effects might disrupt the health of the ecosystem exposed to the MME and, if at a great enough magnitude, could have widespread consequences including loss of species or impacted food sources for top predators, including humans. It is because of the implications of such documented impacts that mining operations in Canada are required to perform Environmental Effects Monitoring (EEM) under the Metal Mining Effluent Regulations (MMER) of the Canadian Fisheries Act in order to determine whether the effluents the mines produce cause adverse effects in the receiving aquatic environment.

1.2 ENVIRONMENTAL EFFECTS MONITORING (EEM)

Environmental Effects Monitoring is a detailed program that includes monitoring effects of complex effluents on fish, fish habitat, and fisheries (Dubé et al. 2002, Dumaresq et al. 2002, Kilgour et al. 2007, Lowell et al. 2002, 2007, Ribey et al. 2002, Weber et al. 2008). Unlike end-of-pipe approaches that do not examine effects in the environment, EEM includes surveying fish and benthic invertebrate communities, as well as performing tissue metal analyses on fish, in order to ensure that receiving environments are protected (Figure 1-1). In addition to field surveys, artificial stream systems that mimic natural systems while controlling water quality, water chemistry, food, and predation have been recognized as an option for monitoring effects of effluents (Dubé et al. 2002, 2006, Hruska and Dubé 2004, 2005). Artifical streams are thus an alternative to monitoring fish and invertebrates in field surveys, and are an accepted component of EEM (Dubé et al. 2002). Allowing for replication in the laboratory or streamside is a primary

benefit of using artificial streams in EEM programs (Culp et al. 1996, Dubé et al. 2002, Lamberti and Steinman 1993). If effects are found from EEM programs (i.e., impacts on fish or benthic invertebrates, or fish tissue contamination), the type of effects must be confirmed and the magnitude of change must be quantified (Figure 1-1).

The EEM program is one of the first regulated programs in Canada that has established decision-making actions on the basis of magnitudes of biological responses. In EEM, a statistical difference in biological samples between reference and effluent exposed sites is considered an effect from the effluent (Hewitt et al. 2003, Lowell et al. 2007, Ribey et al. 2002). Recent EEM program results suggest that MMEs tend to have inhibitory effects, including thinner, slower growing fish with decreased liver and gonad sizes, as well as altered age class structures (Environment Canada 2012). Altered community and species richness structures in benthic invertebrates have also been reported (Environment Canada 2012, Lowell et al. 2007). Many of the inhibitory responses that result from MME exposures have the potential to impact the fitness of those species present in the receiving aquatic environments, which suggests that reproductive impacts should be closely monitored, as well. Although EEM has resulted in a structured national assessment of mine effluents, it is still primarily a monitoring system with focus on the effects of effluents. As mines advance through the phases of EEM, more causal investigations are expected. Understanding modes of action of metals in the environment will improve development of effluent treatment technologies and lead to better understanding of food-web interactions.

1.3 PROCESS OF EXAMINING CAUSAL FACTORS

If an effect from an effluent is confirmed through the EEM program and causal factors are unknown, an investigation of cause (IOC) approach may subsequently be followed. The IOC

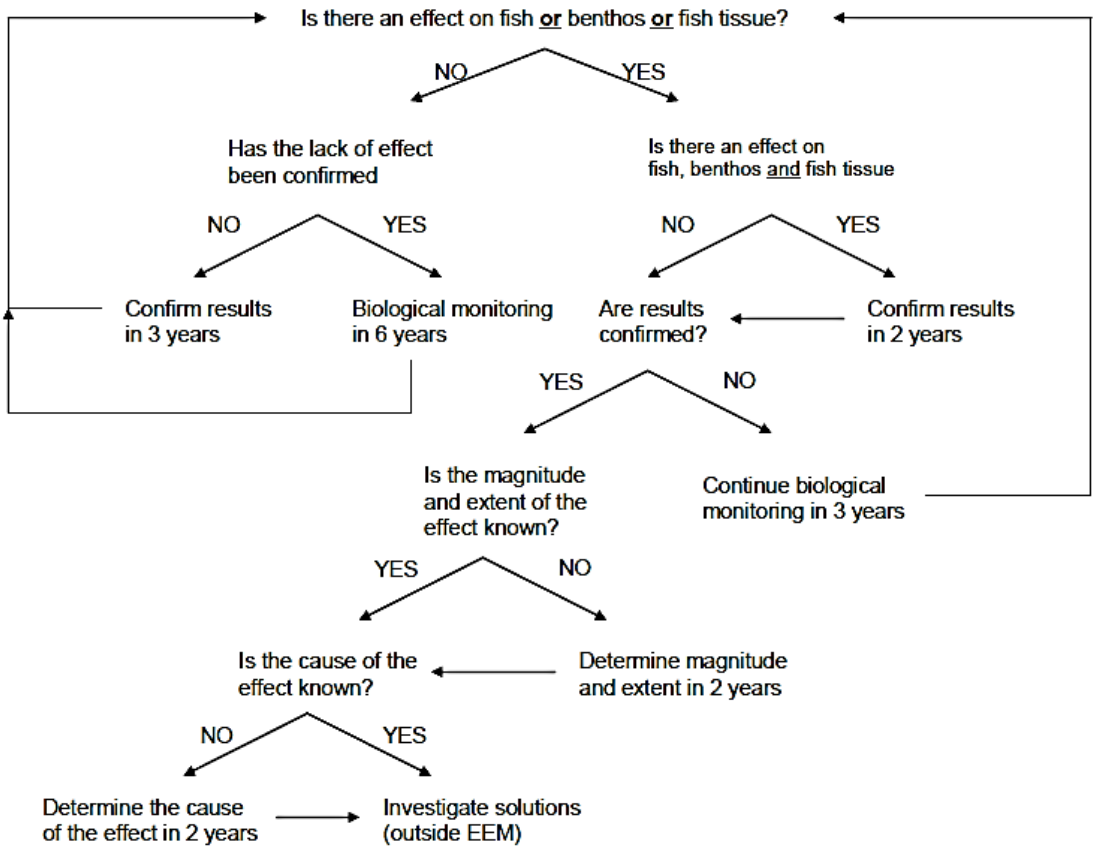

Figure 1-1 – Flow chart from the Environmental Effects Monitoring (EEM) program describing the process of confirming effects, quantifying the magnitude of effects, and investigating the cause of effects from effluents that are released into the environment (from Metal Mining EEM Review Team 2007).

approach consists of confirming and identifying the source of the effects, and identifying and characterizing the chemical(s), compound(s) or metal(s) responsible (Hewitt et al. 2003, 2005), with the purpose of eliminating or minimizing the effects when deemed necessary (Metal Mining EEM Review Team 2007). The initiation of IOC is dependent on the magnitude and extent of any effects, and whether these effects are greater than what was anticipated in any preliminary environmental assessments (Metal Mining EEM Review Team 2007). As conclusively establishing causes of effects can be difficult, the EEM framework recommends using a weight-of-evidence approach, coupled with ecoepidemiological criteria (Hewitt et al. 2003). Associations between cause and effects should be strong, consistent, and specific, and should be coupled with observational and experimentation analyses (Hewitt et al. 2003). In cases when MMEs are found to cause effects, determining specific causes can also be complex due to the number of elements and compounds in the effluents, variability among effluents (and even within an effluent over time), and the numerous factors that can modify toxic responses.

1.4 EFFLUENT COMPLEXITY

Determining specific causes of environmental effects from treated MMEs, such as those described in section 1.1 and 1.2, are difficult due to the complexity of these effluents. Effluents contain a mixture of metals, metalloids, ions, minerals and other contaminants, typically at low pH, with high water hardness, and also with high concentrations of sulphate. Many of the aforementioned parameters, in addition to others that are effluent specific [e.g. concentrations of dissolved organic carbon (DOC)] can vary over time within a particular effluent. The chemical characteristics of the effluents influence metal bioavailability and uptake, and ultimately whether the effluent causes toxicity (Lottermoser 2007). Additionally, some metals that can be found in effluents are more likely to contribute to toxic effects than others.

1.4.1 Elements of concern in metal mine effluents

Many types of elements are present in MMEs. The MMER of the Canadian Fisheries Act includes limits for concentrations of arsenic, lead, nickel, and zinc that can be present in mine effluents (Metal Mining EEM Review Team 2007). However, other elements such as cadmium and selenium, along with copper, nickel, zinc, and lead are often present at elevated concentrations in MMEs and are believed to contribute to toxicity or physiological changes in fish (Levesque et al. 2003, Munkittrick et al. 1991, Muscatello et al. 2008, Pyle et al. 2005). Copper, nickel, selenium, zinc, and arsenic are considered essential or beneficial elements, and are required for normal cell functions and metabolism (Amiard et al. 1987, Bury et al. 2003, Chowdhury et al. 2008, 2008, Niyogi et al. 2006, 2007). However, when essential elements occur above physiological threshold levels they are known to produce toxic responses (Bury et al. 2003). Cadmium and lead are non-essential elements, which have no function in organisms, and can bioaccumulate as a result of pollution and elicit toxic responses (Amiard et al. 1987). The toxic effects of all of the above elements in aquatic animals, especially fish, have been studied in some detail (see Table 1-1 for reproductive effects of copper, nickel, and selenium). However, it is important to note that almost all of these findings are based on studies with single metal exposures that have been performed at greater concentrations or under water chemistry conditions that were quite different (e.g, lower water hardness) than what is present in many MMEs. Furthermore, effects observed in single metal exposures are not always predictive of effects of MMEs (a complex mixture of metals) in fish.

1.4.2 Exposure to multiple metals (metal mixture)

Although effects have been reported to occur from single metal exposures, these types of exposures generally ignore the complexity of MMEs. Individual metals can be present at

Table 1-1 – A summary of the reproductive effects that have been documented in various species of fish due to waterborne or dietary exposure to copper, nickel, or selenium. Effects from each study are presented along with the associated concentrations, pH, and hardness conditions.

Metal	Reproductive Effects	Conc.	Mean pH	Mean Hardness (as $CaCO_3$)	Reference
Cu	Decreased fecundity of fathead minnows (*Pimephales promelas*)	≥34 µg/L[w]	7.9	198 mg/L	Mount 1968
		≥18.4 µg/L[w]	7.2	31.4 mg/L	Mount and Stephan 1969[a]
		≥37 µg/L[w]	7.8	204 mg/L	Gekler et al. 1976
	Decreased fecundity of bluntnose minnows (*Pimephales notatus* Rafinesque)	≥18 µg/L[w]	7.9-8.3	172-230 mg/L	Horning and Neiheisel 1979
	Decreased gamete viability and hatch success of brook trout (*Salvelinus fontinalis*)	≥32.5 µg/L[w]	6.9-8.0	45.4 mg/L	McKim and Benoit 1971
	Decreased hatch success in steelhead trout (*Salmo gairdneri*)	≥31 µg/L[w]	7.4-7.9	120 mg/L	Seim et al. 1984
Ni	Decreased fecundity of fathead minnows	≥0.73 mg/L[w]	7.5-7.9	193-228 mg/L	Pickering 1974
	Decreased hatch success in zebrafish (*Brachydanio rerio*)	≥40 µg/L[w]	7.5-7.7	100 mg/L	Dave and Xiu 1991
	Damage to ovaries of hardy fish (*Oreochromis mossambicus*)	≥0.5 mg/L[w]	n/a	n/a	Siosin and Herrera 2007
Se	Loss of species due to reproductive impairment	≥10 µg/L[w,d]	6.6-7.8	~33-43 mg/L	Lemly 1985
	Abnormal larval development or deformities in northern pike (*Esox lucius*)	≥31.28 µg/g dry weight in eggs[m]	n/a	n/a	Muscatello et al. 2006
	Abnormal larval development or deformities in rainbow trout (*Oncorhynchus mykiss*)	≥8.8-10.5 µg/g wet weight in eggs[m]	n/a	n/a	Holm et al. 2005
	Increased larval deformities and decreased hatch success in fathead minnows	≥9.0 µg/L[w,d,m]	6.5	~457 mg/L	Driessnack et al. 2011[b]

n/a – not available

[a] survival of mature fish was also reduced

[b] exposures were done from an effluent metal mixture, therefore effects might be from a combination of metals

[w] indicates waterborne exposure; [d] indicates dietary exposure; [m] indicates maternal transfer

elevated concentrations in the environment, however it is more likely that a combination or mixture of elements will instead be elevated. Determining effects from mixtures is challenging, particularly considering the fact that there can be additive, less than additive, or more than additive effects. Additive effects occur when the effects from the mixture equal the expected effects from each individual metal summed together (Norwood et al. 2003). More than additive effects occur when the mixture becomes toxic due to the combination of metals that are not toxic alone or when toxicity is greater than expected following the mixture of toxic metals (Norwood et al. 2003). Less than additive effects occur when the mixture is less toxic than it would be as individual metals or less toxic what should be expected when considering the toxicity of the individual metals summed together (Norwood et al. 2003).

Generally, studies have not examined whether specific responses can be attributed to the presence of individual metals or the combination of metals. For example, would similar reproductive effects occur if the MME consisted only of one metal, or are multiple metals required to produce effects? Moreover, are the multiple metal exposure effects additive or antagonistic in nature? Understanding whether or not similar effects occur in mixed metal exposures relative to single metal exposures is required in order to proceed with finding factors that can modify toxicity and lead to appropriate treatment options.

1.5 INDIRECT EFFECTS

In addition to direct toxic effects, effects on one group of organisms or species might indirectly contribute to effects on another group of organisms or species. Indirect effects are generally food-based, where an effect on a prey species is likely to contribute to negative effects in its predators (Campbell et al. 2003, Rasmussen et al. 2008). For example, metal contamination has been shown to affect the densities of zoobenthic communities in lakes, thus

shifting the feeding patterns and reducing the growth of yellow perch (*Perca flavescens*) (Iles and Rasmussen 2005, Kövecses et al. 2005). Reduced *Chironomus dilutus* densities have also been reported due to metal mine effluent exposures (Hruska and Dubé 2004, 2005). Reductions in food availability could contribute to reductions in fecundity of fish, as individuals that consume higher quality and quantity of food generally reproduce at a higher rate than individuals who do not consume sufficient quantities of food (Caceres et al. 1994). Fitness of fish might also be affected due to consumption of contaminated food (i.e. food quality), although the degree of effect would depend on the quantity of food and specific metals being consumed.

Although indirect effects can play a significant role in influencing responses to environmental contaminants, their effects are generally less well-studied than direct responses. Most studies that have examined indirect effects of altered food structures on fish have been performed in the natural environment (Iles and Rasmussen 2005, Kövecses et al. 2005, Munkittrick and Dixon 1988, Munkittrick et al. 1991), and although they include potentially important shifts in diet due to relevant food-web modification, they also tend to lack the ability to separate direct effects of metal contamination on fish from the indirect effects of food-web changes (Rasmussen et al. 2008). Furthermore, field studies include tolerant species, such as yellow perch or individuals that have developed resistance to the contamination over time (Rasmussen et al. 2008). Thus, it is important to examine the role of food quantity in influencing metal bioavailability and toxicity in MMEs in a controlled laboratory setting in order to better understand indirect toxic effects in the environment under varying food availability, alongside the direct effects of metal contamination.

1.6 BIOAVAILABILITY AND TOXICITY MODIFYING FACTORS

Although concentrations of metals in an aquatic ecosystem might be elevated, those metals must be available to fish for toxicity to occur. In the case of MMEs, the presence of elevated metal concentrations might not be a contributing cause of toxicity if the metals are not available for uptake in the resident biota. The routes of uptake (via water and/or diet) and water chemistry each play an important role in influencing metal bioavailability and bioaccumulation in the aquatic organisms.

1.6.1 Routes of uptake

Ultimately, metal uptake and toxicity in fish depends on bioavailability through water or diet. Metals can be taken up by fish through gills (waterborne uptake) and/or the gut (dietary uptake) and can be distributed to tissues throughout the body (see Bury et al. 2003, and Dallinger et al. 1987 for reviews). Generally, metal uptake can occur through both the water and diet if the metals are bioavailable. However, the diet can be a more important source of uptake for some elements, such as selenium, where accumulation in fish occurs primarily as a result of dietary consumption (Hamilton 2004, Muscatello et al. 2008). Once a metal enters the body of a fish, accumulation tends to occur in the gills, liver, body tissue, or kidneys (Kamunde and Wood 2004), although some metals (e.g., selenium) can accumulate in the ovaries of fish and be transferred maternally to the offspring (Muscatello et al. 2006).

1.6.2 Water chemistry

Aquatic metal bioavailability is inherently linked to water chemistry. Water chemistry parameters, such as hardness, pH, alkalinity, and natural organic matter (NOM) influence metal speciation and bioavailability, therefore these parameters also influence bioaccumulation and toxicity. In general, for metals to be available and toxic to biota in an aquatic ecosystem, the free

metal ions must be available to bind to biotic ligands in the biota (i.e., the metals must be bioavailable) (Di Toro et al. 2001). However, competition and complexation among free metal ions and cations, inorganic ligands, and organic matter influence free metal ion bioavailability (Figure 1-2). Complexation can affect the species of metals present in the water (i.e., metal speciation). Complexation between free metal ions and either organic matter or inorganic ligands can reduce the availability of the free metal ions to the biotic ligands, and can thus decrease toxicity to biota. Free metal ions also compete with other cations that are present in the water (e.g., calcium, magnesium, or hydrogen ions) for binding sites on the biotic ligands. If the concentration of natural cations greatly exceeds the concentrations of free metal ions, the uptake of free metal ions will be decreased due to increasing competition, and as a consequence toxicity will decrease. However, some water chemistry parameters may be more influential to toxicity of metals than others, and not all metals or metalloids are affected in the same way or by the same degree. In metal mixtures, water chemistry will influence each metal in a specific manner, and individual metals may interact or interfere with each other which make predictions complicated (Norwood et al. 2003). In addition, the current understanding of the influence of water chemistry on metal availability to the aquatic biota are mainly based on short-term acute metal exposures, and whether water chemistry can modulate metal bioavailability and toxicity in the same manner under the chronic exposure conditions remains to be fully understood.

1.6.2.1 Water hardness

Water hardness is perhaps the most important water chemistry parameter that can influence metal toxicity. As a result, many government regulatory agencies set the maximum allowable metal concentrations based on site-specific water hardness, which includes calcium and magnesium concentrations (expressed as $CaCO_3$). For example, concentrations of cadmium,

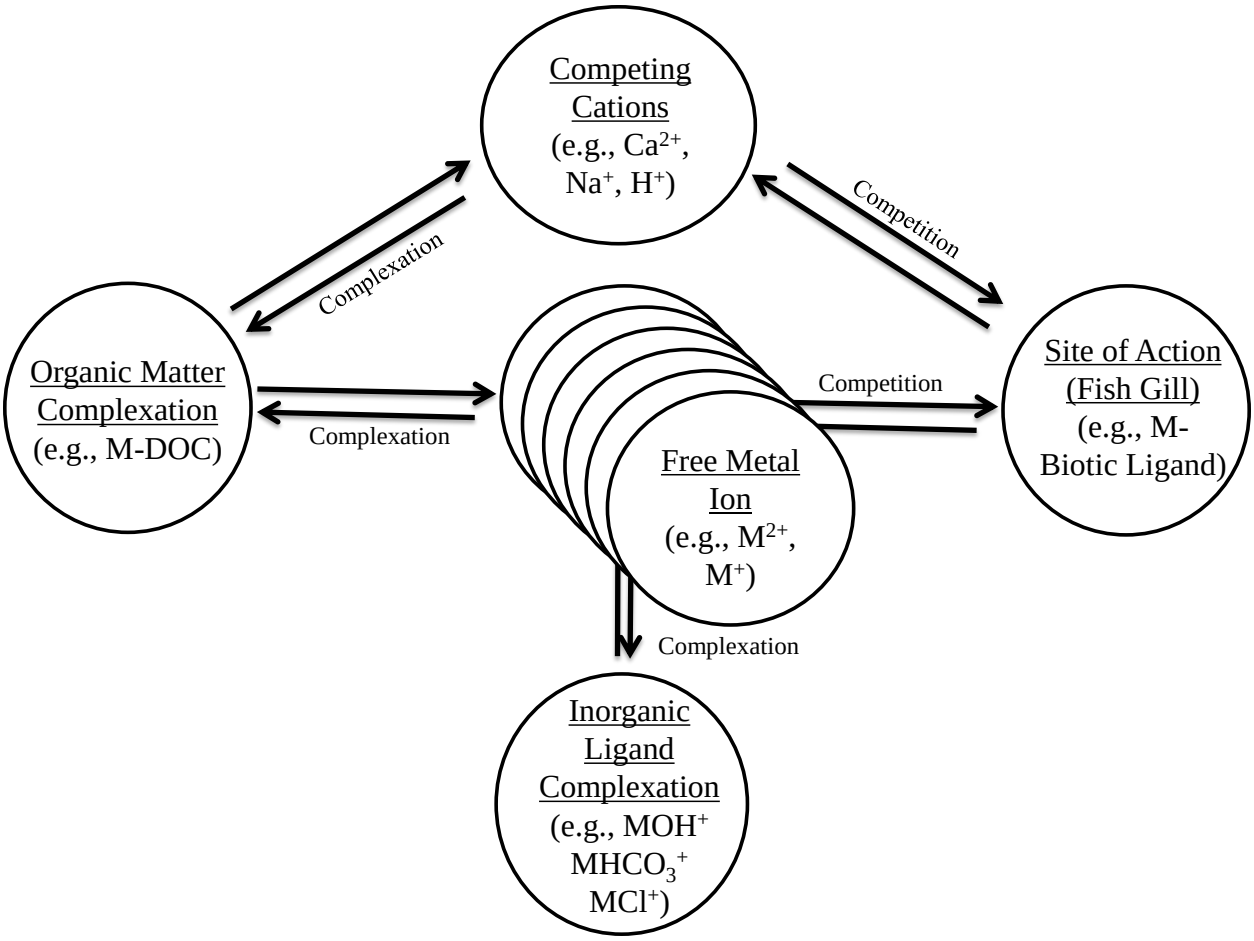

Figure 1-2 – Schematic diagram demonstrating the complexation and competition of free metal ions to inorganic ligands, organic matter, or competing cations, each of which influence the availability of metals to the biotic ligand of fish during waterborne metal exposures {modified from Di Toro et al. 2001).

copper, lead, and nickel are regulated in Canadian waterbodies based on water hardness (CCME 2008). In general, water hardness has been shown to decrease toxicity because the increased number of cations associated with water hardness (Ca^{2+} and Mg^{2+}) compete with metal cations for binding sites on the organism. As a result, water hardness can decrease the bioavailability of metals and thus decrease toxicity. Many studies have demonstrated the reduction in metal accumulation and toxicity in fish as a result of the presence of water hardness cations, including a decrease in gill accumulation of cadmium and toxicity in rainbow trout (*Oncorhynchus mykiss*) (Niyogi et al. 2008), as well as a decrease in acute nickel toxicity in larval fathead minnows (*Pimephales promelas*) (Pyle et al. 2002), to name a few. Because of the role of water hardness in potentially regulating metal toxicity, MMEs with high concentrations of calcium and/or magnesium might be less toxic than the same MME containing low concentrations of calcium and/or magnesium. However, the majority of single and mixed metal exposure studies with fish have been conducted in waters with hardness concentrations less than 200 mg/L (see Table 1-1), which might not be representative of the conditions present in MMEs. The elevated hardness of some MMEs could mitigate a number of the effects observed in previously studied single metal exposures.

1.6.2.2 Natural organic matter, alkalinity, and pH

NOM, alkalinity, and pH affect metal bioavailability by influencing metal speciation and complexation, thereby also influencing toxicity. Increases in NOM [typically measured as dissolved organic carbon (DOC)] have been linked to lower metal toxicity as a result of increased complexation, and therefore, reduced uptake of toxic free metal ions (Niyogi and Wood 2004, Playle et al. 1993, Wood et al. 2011). If the metal ions bind more strongly to the NOM than to the fish gill (i.e. biotic ligands), then free metal ions will be less bioavailable. The

toxicity of copper, zinc, and lead for example, has been shown to decrease due to the presence of NOM in several fish species during acute exposures (e.g., Buckley 1983, Clearwater et al. 2002, Hutchinson and Sprague 1987, Zitko et al. 1973). Increased DOC might also reduce metal bioaccumulation in fish during chronic exposures, as Mager et al. (2010) reported a decrease in chronic lead toxicity due to the presence of increased DOC. As a result, increasing DOC in MMEs could potentially be a method for reducing toxic effects and an acceptable MME treatment option. However, it has also been suggested that DOC might not offer the same degree of protection, as observed under acute exposures, during chronic exposures to metals (Brauner and Wood 2002a). Therefore, further studies exploring the role of DOC during chronic exposures with complex metal mixtures must still be performed.

Increased alkalinity has also been linked to a decrease in toxicity of copper to fish, although this decrease could also be due to increased pH (Van Genderen et al. 2008). Increased pH tends to occur with increased alkalinity, and therefore may also be associated with metal complexation (Niyogi et al. 2008). Metal toxicity tends to increase at low pH where metal complexes dissociate causing metals to occur in their free ion forms that are most bioavailable and toxic (Campbell and Stokes 1985). However, at low pH, the increased H^+ ion concentrations can also cause decreased metal bioavailability through increased competition (Campbell and Stokes 1985, Meador 1991). Therefore, the effects of pH depend on the metal. For example, in hard water (300-320 mg/L as $CaCO_3$), Cu and Pb were the most toxic to four different species (*P. promelas*, *Ceriodaphnia dubia*, *Hyalella azteca*, and *Lumbriculus variegatus*) at pH 6.3, however Cd, Ni, and Zn were most toxic at pH 8.3 (Schubauer-Berigan et al. 1993). Just as NOM could potentially be used as a treatment option for MMEs, elevated alkalinity could also be a possible method for reducing toxicity of MMEs, particularly when alkalinity is lower in the

MME than the receiving environment following the effluent treatment process. However, the success of alkalinity modifications may depend on the metal composition of the MME.

The importance of water chemistry parameters, such as NOM, alkalinity/pH, and hardness on metal accumulation and toxicity cannot be overstated. These variables, particularly alkalinity and NOM, need to be explored with metal mine effluents in order to understand how they influence metal bioavailability in complex metal mixtures, determine whether they can be manipulated in order to decrease metal toxicity of MMEs, and ultimately provide additional treatment options for metal mining operations. Although some studies have evaluated the roles of alkalinity/pH and NOM under acute conditions, virtually no studies have examined the influence of these parameters under chronic conditions with environmentally relevant metal mixtures or MMEs.

1.7 METAL MINE EFFLUENTS IN THE ENVIRONMENT

Although many MMEs have similar characteristics, they are still independent of one another and may elicit very specific responses. The research performed in this thesis is based on a specific MME, released into Junction Creek, Ontario, which has been shown in several past studies to cause reproductive changes in fathead minnows despite unknown causal factors (see section 1.7.2). This MME has a variety of metals present at elevated concentrations, and therefore it is an ideal source of an environmentally relevant metal mixture for performing causal investigations, water chemistry manipulations, and examining direct and indirect effects during chronic exposures.

1.7.1 Junction Creek watershed

The Junction Creek watershed is located in Sudbury, Ontario, Canada (Figure 1-3). This

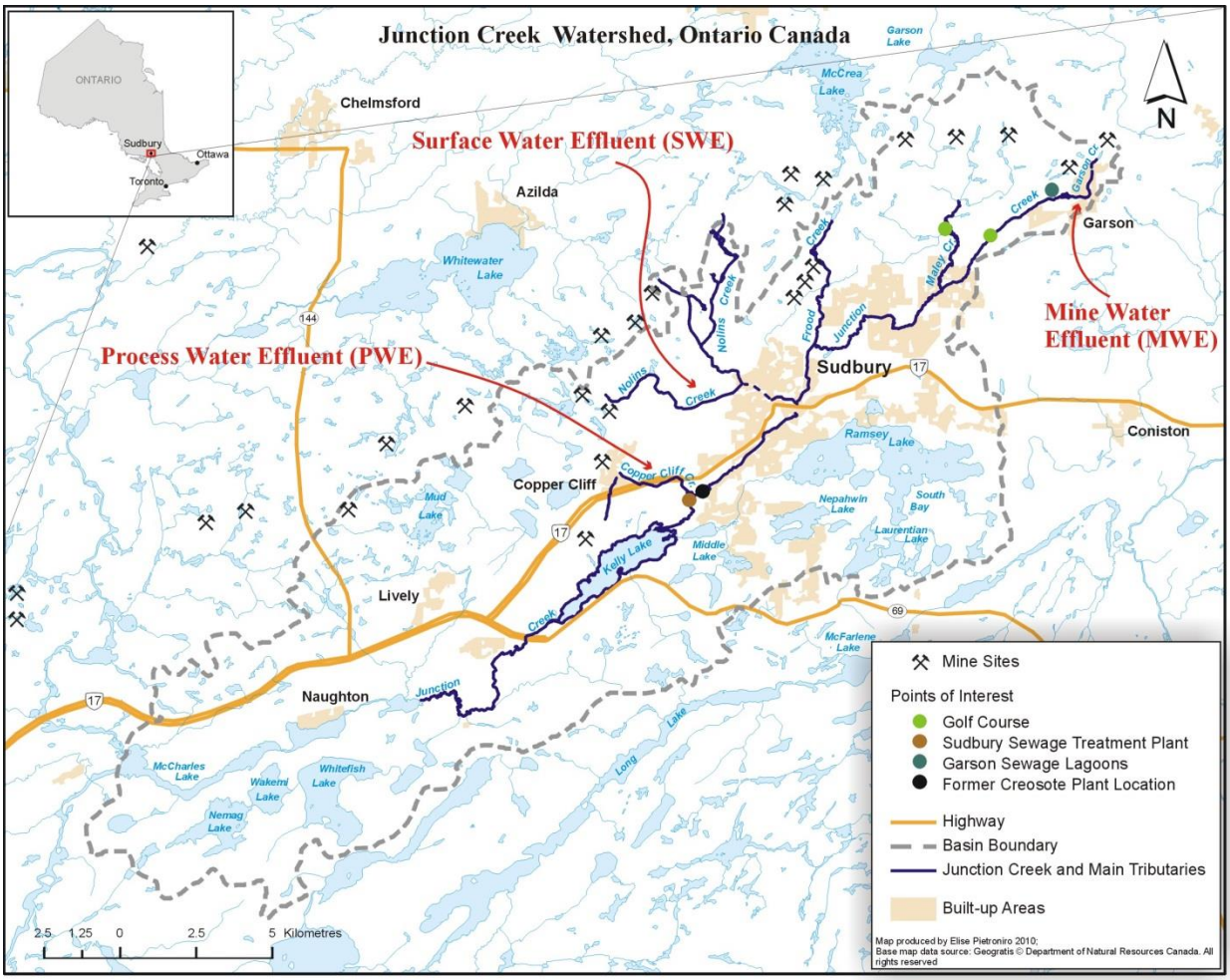

Figure 1-3 – Map of metal mine effluent discharge sites in the Junction Creek watershed and its tributaries, near Sudbury, Ontario, Canada (reproduced with permission from Rozon-Ramilo 2011). Water in the Junction Creek flows southwest and enters Kelly Lake.

area contains large deposits of metals that have been mined for over 100 years (Rickwood et al. 2008, Weber et al. 2008). Currently, Vale Ltd., which produces copper, nickel, precious metals, platinum-group metals, sulfuric acid, and liquid sulfur, operates several mines in this area and Junction Creek is the recipient of the resulting effluents (Rickwood et al. 2008).

Junction Creek receives treated metal mine effluents from three separate discharges, mine water effluent (MWE), surface water effluent (SWE), and process water effluent (PWE), in addition to municipal wastewater and runoff (Rickwood et al. 2008, Weber et al. 2008). Effluent treatment from these mines consists of addition of lime and settling of precipitates, along with subsequent pH adjustments (Rickwood et al. 2006a). Average daily flow rates of treated effluents from MWE, SWE, and PWE into Junction Creek from 2002 to 2008 were 2659 m^3/day, 16727 m^3/day, and 100220 m^3/day, respectively (Source: Vale Ltd.). Estimated effluent concentrations where the respective creeks flow into Junction Creek are 20% for MWE, 30% for SWE, and 45% for PWE (Dubé et al. 2006). The analysis of metal mine effluents has shown elevated concentrations of barium, boron, cobalt, copper, lithium, nickel, rubidium, selenium, strontium, and thallium in all three MMEs (Rozon-Ramilo et al. 2011a). A municipal wastewater discharge is also approximately 200 metres downstream from the PWE discharge and releases treated sewage at a rate of approximately 102000 m^3/day.

Historical contamination in the Junction Creek area is known to be a source of environmental concern. Soils and sediments in the Junction Creek watershed contain high levels of metals from past mining and milling operations, while previous ecological damage has been observed in the surrounding areas (Dubé et al. 2006, Pyle et al. 2005, Rickwood et al. 2008, Weber et al. 2008). There are 12 known species of fish present in the Junction Creek watersheds, including creek chub (*Semotilus atromaculatus*), pearl dace (*Semotilus margarita*),

and fathead minnow, and possibly two additional unconfirmed species [finescale dace (*Phoxinus neogaeus*) and blacknose shiner (*Notropis heterolepis*)] (Lemieux et al. 2004). The diversity and density of fish and invertebrates is lower downstream from the MME discharge points in Junction Creek (Jaagumagi and Bedard 2002).

1.7.2 MME research in Junction Creek

Research on the effects of MMEs that are present in Junction Creek has been ongoing since 2001. Studies have included field sampling surveys (Weber et al. 2008), laboratory and field studies using water-only exposures (Dubé et al. 2006) and multi-trophic exposures (i.e., waterborne and dietary) (Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a) in order to assess effects in fish and invertebrates exposed to MMEs in Junction Creek. Following the studies that assessed whether or not effects occur from MME exposures, the role of food quality in influencing toxic responses in fathead minnows exposed to MME was examined (Rozon-Ramilo et al. 2011b) in order to move towards causal investigations.

Within the Junction Creek watershed, fish species, such as fathead minnows and creek chub, have been collected that had elevated tissue concentrations of Cd, Cu, Rb, Se, and Sr (Weber et al. 2008). Weber et al. (2008) reported that the body weights and liver sizes of collected fish were significantly influenced by the site of collection and the presence of elevated concentrations of metals in the water. However, effects were often observed in areas of both municipal sewage and effluent discharge. Therefore, MMEs, historical metal contamination, urban runoff, or the sewage treatment plant could have been the source of the effects. In order to isolate the MMEs from confounding environmental factors, several mesocosm studies were performed by collecting MMEs prior to discharge into the environment. These studies found that 45% PWE typically contains the highest concentrations of metals of the MMEs released into

Junction Creek and contributes to metal accumulation in fish tissues and decreased reproductive performance in fathead minnows (Dubé et al. 2006, Rozon-Ramilo et al. 2011a). Furthermore, it was reported that the MWE and SWE did not impair fathead minnow reproduction during the 21-day multi-trophic exposures (Rozon-Ramilo et al. 2011a). It has also been determined that the presence of MME contaminated food sources (i.e. *C. dilutus*) increases toxic responses in fathead minnows during chronic exposures to the 45% PWE. Specifically, differences in egg production were greater between control and treatment fish when exposed to PWE through the water and diet, relative to differences in egg production when exposures were performed through the water only (Rickwood et al. 2006a). Additionally, fathead minnow responses have been shown to differ depending on whether they consume contaminated or uncontaminated *C. dilutus* larvae in both uncontaminated and PWE contaminated water. Specifically, fathead minnows in 45% PWE that had been fed *C. dilutus* larvae cultured in 45% PWE had lower egg production than fathead minnows in control water that had been fed either *C. dilutus* larvae cultured in 45% PWE or cultured in control water (Rozon-Ramilo et al. 2011b). Fathead minnows in 45% PWE that had been fed *C. dilutus* larvae cultured in control water also had greater egg production than the fathead minnows exposed to 45% PWE through both the water and diet (Rozon-Ramilo et al. 2011b). Overall, these studies suggest that 45% PWE causes decreases in fathead minnow egg production and that dietary contamination is likely a major contributing factor in addition to contaminated water. However, the specific causes of toxic effects reported in previous studies have yet to be investigated in detail.

1.8 SUMMARY OF RESEARCH GAPS

Although previous studies have consistently reported several metals to be present at elevated concentrations in MMEs, the same metals have not always been found to accumulate in

the fish. This suggests that some metals are not available for uptake or are simply not transferred to the fish tissues. Research is needed to determine whether individual metals, for example Cu, Ni, and Se, which are toxic at fairly low concentrations and typically elevated in MMEs (PWE included) and fish tissues, result in the same effects on trophic systems as do whole-effluents. These metals have also been reported to contribute to reproductive effects (see Table 1-1), which are comparable to reproductive effects caused by 45% PWE. Additionally, since diet has been shown to be an important factor in the transfer and accumulation of certain metals in fish, and since food quality has previously been examined, the role of food quantity in influencing bioaccumulation and reproduction should be explored to further evaluate the role of diet in toxic responses during chronic MME exposures. Lastly, since water chemistry parameters such as NOM and alkalinity are believed to be important modifying factors of metal bioavailability and toxicity to aquatic organisms, their roles in influencing MME toxicity should be examined. Altogether, determining what metals (either singly or in mixture) are the primary drivers of toxicity, exploring the role of indirect factors such as food availability (i.e., diet quantity) in trophic transfers of metals and influencing toxicity, and understanding how metal bioavailability and toxicity in fish are influenced by water chemistry will allow for the development of improved treatment options for metal mines.

1.9 THE FATHEAD MINNOW REPRODUCTIVE TEST

The fathead minnow reproductive test was utilized for each of the studies presented in this thesis (Ouellet et al. 2013a, 2013b, 2013c). The studies presented in Chapters 2 and 4 were performed using multi-trophic mesocosms with fathead minnows and live *C. dilutus* larvae as the food source. The study in Chapter 3 was performed with fathead minnows and frozen *C. dilutus* larvae, although it was performed in the same mesocosm streams and under similar conditions to

the other studies. All research was performed at the University of Saskatchewan in Saskatoon, SK, Canada.

1.9.1 General methodology – Mesocosms

The artificial stream mesocosms used in all studies presented in this thesis are an accepted component of Canada's EEM program. These streams have been utilized in a number of past studies that have evaluated reproductive effects in fish, and bioaccumulation of metals from MMEs to target organisms/tissues (e.g., *Chironomus* spp., fathead minnow liver, gonad, gill, and carcass tissues) in Ontario (Dubé et al. 2006, Hruska and Dubé 2004, 2005, Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a, 2011b), New Brunswick (Dubé et al. 2005), Saskatchewan (Driessnack et al. 2011), and the Northwest Territories (Spencer et al. 2008). The mesocosms are made up of individual tables that hold up to 8 replicate, 10.3-L circular polyethylene artificial streams in order to expose fish to a particular treatment (see Figure 1-4). The replicate streams are fed from an 8-port manifold under continuous, recirculating flow, providing consistent water exchange and flow in each replicate stream. Each mesocosm table represents a single treatment. Furthermore, the mesocosms allow for isolated waterborne exposures, or simultaneous waterborne and dietary exposures, typically over a period of 21 days, as a means of assessing chronic effects of MMEs on both fish and invertebrates.

1.9.2 Fathead minnow overview and biology

The fathead minnow (*P. promelas*) is part of the family Cyprinidae and is a ray-finned, bony fish that is distributed throughout North American waterbodies. Adults live for approximately 2 years (Hartviksen and Momot 1989) and are sexually mature by the age of 6 months (Ankley et al. 2001). Mature female fathead minnows typically weigh 2-3 grams, while

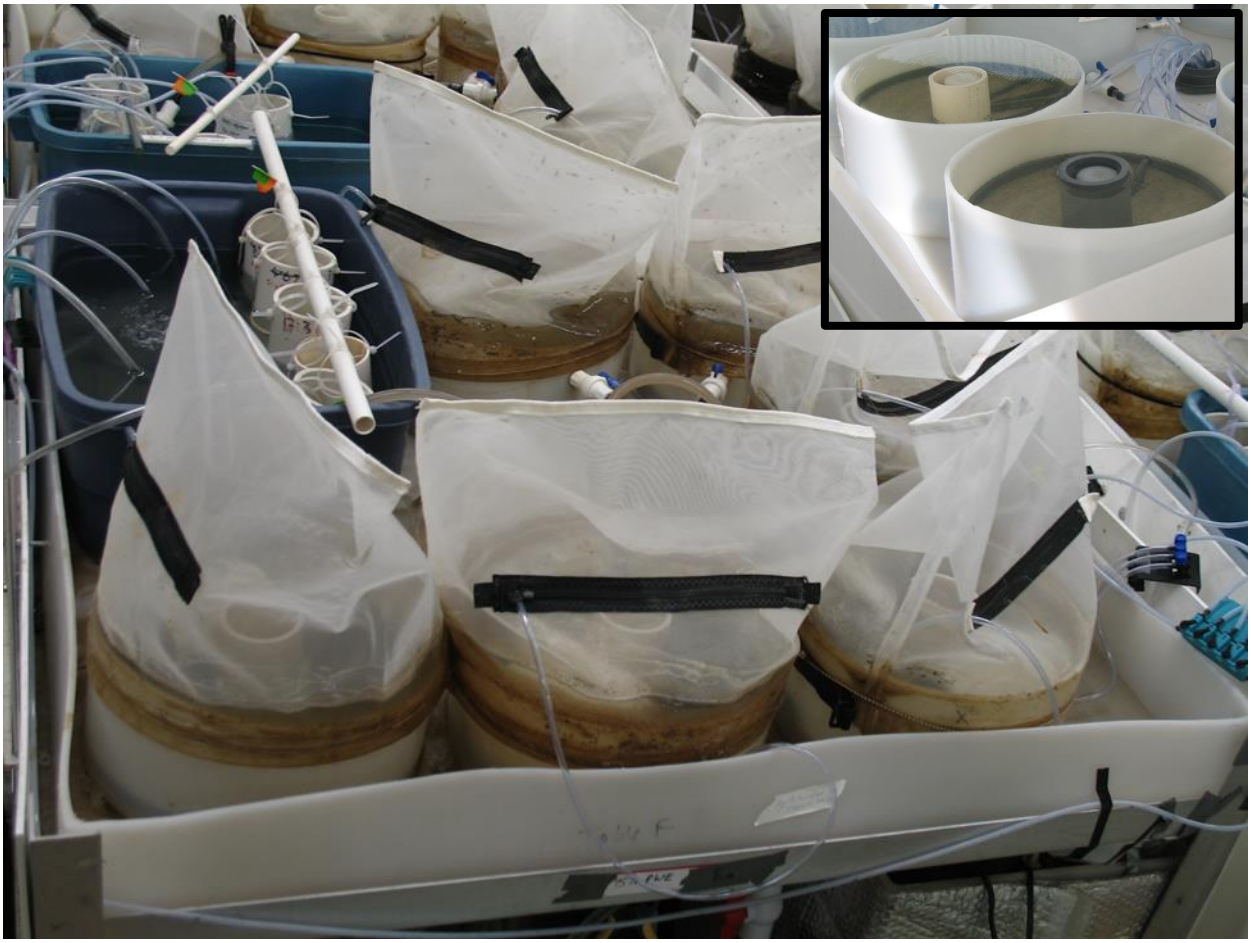

Figure 1-4 – An example of six multi-trophic mesocosm streams (1 treatment with 6 replicate streams) with screen covers to prevent emerged *C. dilutus* from escaping, along with larval cups (top left) for larval *P. promelas* hatching. Two streams with feeding barriers are also shown, top right, without the screen covers.

mature male fathead minnows are usually larger than females and weigh 4-5 grams (Ankley et al. 2001). Although immature males and females are nearly identical in appearance, sexually mature males develop a black, fleshy pad that extends from the nape to the dorsal fin, cranial nuptial tubercles, and black vertical bands along their sides. Mature females develop an ovipositor (Ankley et al. 2001) (Figure 1-5).

Fathead minnows have been used in toxicological testing since the 1950s and are commonly used for regulatory and experimental applications (Ankley and Villeneuve 2006). As such, standardized test procedures are well-developed and followed in the research present in this thesis. The fathead minnow reproduction test is recommended for toxicological testing when population level impacts are the relevant endpoint (Ankley and Villeneuve 2006). Predictable breeding behaviour is a major factor for the use of fathead minnows in reproductive toxicology tests, which consists of using sexually mature fathead minnows with fecundity as a primary endpoint. Fecundity is measured by egg production, fertilization rate, and hatch success. Generally, a breeding pair of fathead minnows produces 50-150 fertilized eggs in 3-4 day intervals and the embryos hatch 4-5 days later (Ankley et al. 2001). As a result, eggs and larvae can be easily counted and monitored for effects. Depending on fertilization rate, a reduction in approximately 50% of fathead minnow egg production is believed to cause dramatic reduction in the population (Miller and Ankley 2004). Relative gonad size, relative liver size, and body condition of fathead minnows are normally measured at the conclusion of the exposure period in order to assess reproductive and energetic impacts (Ankley et al. 2001, Ankley and Villeneuve 2006).

Typically, the fathead minnow reproduction test includes a pre-exposure phase that ranges from 7-21 days (to assess baseline egg production), along with a 21-day exposure period (to

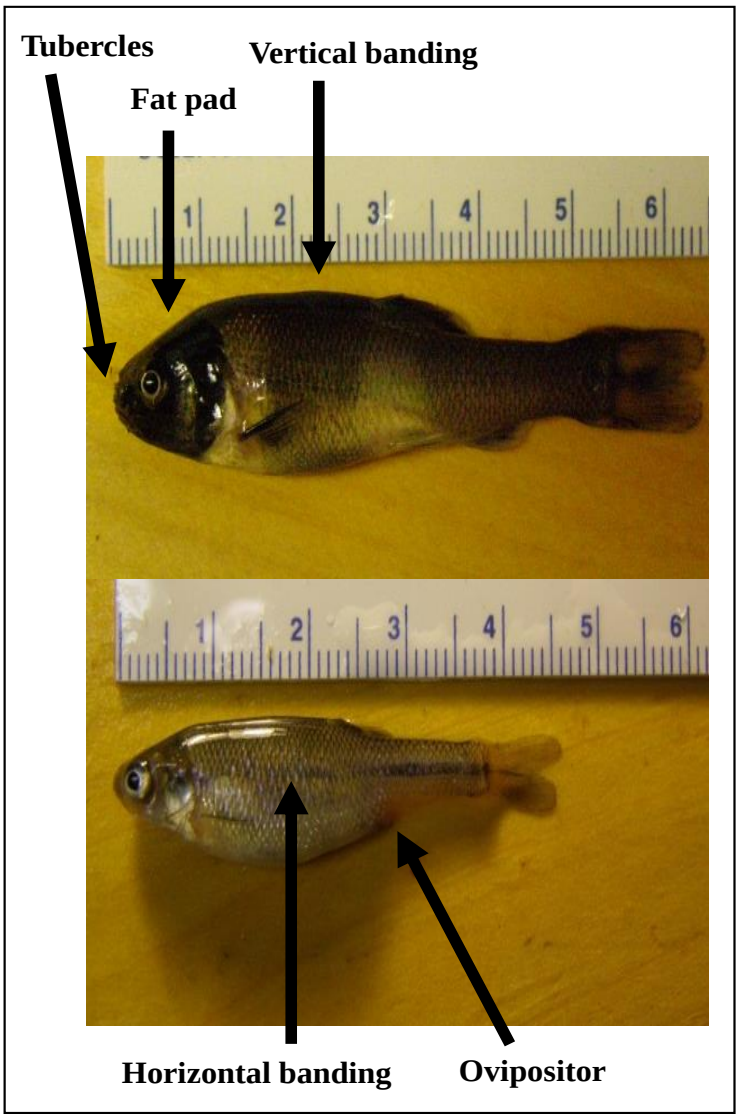

Figure 1-5 – Sexually mature male (top) and female (bottom) fathead minnows (*P. promelas*). Characteristics of sexually mature male fathead minnows include nuptial tubercles, a fleshy fat pad, and vertical banding. Characteristics of the sexually mature female fathead minnow include a horizontal band and an ovipositor. Ruler measurements are presented in cm.

determine reproductive output during treatment). Experiments can include the use of breeding groups of four females and two males, as recommended by Ankley et al. (2001), however pair-breeding with one female and one male as the replicate has been used when linking specific effects to specific fish are required (e.g., Driessnack et al. 2011, Rickwood et al. 2006a, 2006b, 2006c, 2008, Rozon-Ramilo et al. 2011a, 2011b, Werner et al. 2010).

1.9.3 Chironomus dilutus biology

The freshwater midge, *C. dilutus* (formerly *C. tentans;* Diptera: Chironomidae), is also commonly used in toxicity and environmental testing because of its well-understood lifecycle, its ability to be cultured in the laboratory, and because it maintains contact to the sediment (a source of contamination) throughout larval development (Benoit et al. 1997). In addition to acquiring contaminants from the sediments, *C. dilutus* larvae can also accumulate contaminants from the water (Stuijfzand et al. 2000). Furthermore, *Chironomus* spp. frequently account for a large portion of benthic invertebrate biomass and are part of the diet of many fish, all of which make them environmentally relevant as a test species, as well as useful in environmental monitoring and trophic-transfer studies (Benoit et al. 1997, Nebeker et al. 1984, Swansburg et al. 2002).

The *C. dilutus* life-cycle is short and includes complete metamorphosis in approximately 23 to 30 days at 23ºC (Benoit et al. 1997). Females produce a single egg mass within one day of mating, and eggs begin hatching within 2 days and take from 2 to 6 days to complete. The larvae develop for approximately 23 days before entering the pupae stage and no longer feed. Pupation lasts from 1 to 2 days and males typically emerge 5 days prior to peak female emergence. Both males and females die within 7 days of emergence. Therefore, mating occurs quickly after emergence.

1.10 RESEARCH OBJECTIVES

The primary objective of this thesis was to examine potential causal factors of reproductive impairment in fathead minnows during MME exposures. I hypothesized that toxic responses in fish during exposures to an MME are influenced by several factors, including metal composition, availability of food in the ecosystem, and water chemistry. An important assumption being assessed in this thesis was that metal bioavailability is responsible for reduced fecundity and physiological changes in fathead minnows during chronic exposures to MMEs, and therefore, both waterborne and dietary exposure routes were incorporated in this research. Specifically, the research aspects that were explored in this thesis were:

i) To examine whether exposure to specific metals that are present in an MME (e.g., Cu, Ni, Se), alone or in combination, are able to contribute to reproductive effects in fathead minnows observed during chronic exposure to the MME (CHAPTER 2)

ii) To evaluate whether direct effects of metal bioaccumulation or indirect effects of differences in food availability influence reproductive impairment in fathead minnows during chronic exposure to an MME (CHAPTER 3)

iii) To assess whether water chemistry modifications ameliorate metal bioaccumulation and reproductive impacts observed in fathead minnows during chronic exposure to an MME (CHAPTER 4).

1.10.1 Specific objectives and hypotheses

Objective 1 (Chapter 2) – To determine whether several metals that are present at elevated concentrations in MMEs, specifically copper, nickel, and selenium (alone or in combination), accumulate in fathead minnow tissues and are responsible for reproductive impairment observed in fathead minnows during exposure to PWE.

Predictions – If copper, nickel, and/or selenium are bioavailable in 45% PWE, then these metals will accumulate in fathead minnow tissues and lead to reduced egg production during exposure to 45% PWE. If these metals alone are responsible for the effects in 45% PWE then exposure to single and mixed metal treatments, under comparable concentrations and water chemistry conditions to 45% PWE, will result in similar metal bioaccumulation patterns and reduced egg production as observed with the 45% PWE exposure.

Null hypothesis – Tissue concentrations of copper, nickel, and selenium will not be statistically different among female fathead minnows following chronic exposure to single or mixed metal treatments of these metals and 45% PWE, and no reproductive impairment will be observed in any treatment.

Objective 2 (Chapter 3) – To determine whether differences in food availability influence metal bioaccumulation and reproduction in fathead minnows during exposure to MME.

Predictions – If trophic transfer of metals through food consumption is responsible for metal bioaccumulation and fathead minnow reproductive impairment, then differences in the quantity of food consumed will lead to differences in metal bioaccumulation and reproduction in fathead minnows. If food quantity alone influences fathead minnow reproduction, then fathead minnows that consume greater quantities of food will also have greater egg production.

Null hypothesis – Concentrations of metals in female fathead minnow tissues and egg production will not be statistically different between treatments of normal food and low food during exposures to 45% PWE.

Objective 3 (Chapter 4) – To determine whether water chemistry parameters, such as alkalinity or NOM, will reduce metal bioavailability and accumulation, as well as reproductive impacts in fathead minnows chronically exposed to 45% PWE.

Predictions – If metal bioaccumulation is responsible for reproductive impairment in fathead minnows during exposure to 45% PWE, then increased alkalinity or NOM will decrease the bioavailability of metals through complexation effects and improve fathead minnow egg production.

Null hypothesis – Concentrations of metals in female fathead minnow tissues and egg production will not be statistically different among 45% PWE treatments with high and low alkalinity or NOM.

1.11 FORMAT OF THE THESIS

This thesis has been organized in a manuscript format for publication in scientific journals. As a result, there may be some repetition of introduction, and materials & methods throughout the thesis.

Chapter 2 has been published in the journal of Water, Air, & Soil Pollution, 2013, volume 224, issue 3: pages 1-19, under joint authorship with Som Niyogi (University of Saskatchewan) and Monique G. Dubé (Canadian Rivers Institute).

Chapter 3 has been published in the journal of Ecotoxicology and Environmental Safety, 2013, volume 91:188-197, under joint authorship with Som Niyogi (University of Saskatchewan) and Monique G. Dubé (Canadian Rivers Institute).

Chapter 4 has been accepted in the journal of Ecotoxicology and Environmental Safety, May 17, 2013, under joint authorship with Som Niyogi (University of Saskatchewan) and Monique G. Dubé (Canadian Rivers Institute).

2 CHAPTER 2[a]

A SINGLE METAL, METAL MIXTURE, AND WHOLE-EFFLUENT APPROACH TO INVESTIGATE CAUSES OF METAL MINE EFFLUENT EFFECTS ON FATHEAD MINNOWS (*PIMEPHALES PROMELAS*)

[a] This chapter examines the potential contribution of several metals (Cu, Ni, and Se), that are usually elevated significantly in a Canadian metal mine effluent, to toxic responses in fish during chronic exposures to the effluent. The purpose of Chapter 2 was to investigate direct factors of toxicity (i.e., specific metals and metal mixtures – potential causative factors of toxicity) and their role in causing metal bioaccumulation and reproductive impairment in fathead minnows during chronic exposure to the metal mine effluent. Chapter 2 has been published in the journal of Water, Air, & Soil Pollution, 2013, 224(3):1-19 under joint authorship with Som Niyogi (University of Saskatchewan) and Monique G. Dubé (Canadian Rivers Institute).

2.1 INTRODUCTION

Metal mine effluents (MMEs) can be a significant source of metals that have the ability to negatively impact organisms in receiving aquatic environments. Many studies have documented effects from exposure to MMEs, particularly to invertebrate and fish populations. For example, reduced benthic invertebrate densities (Hruska and Dubé 2004), and reproductive changes (e.g., altered egg production, larval deformities, egg sizes, growth) (Driessnack et al. 2011, Jezierska et al. 2009, Muscatello et al. 2006, Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a, 2011b), altered gill functions and hormone levels (Levesque et al. 2003), cellular changes (Payne et al. 2001), as well as increased tissue metal burdens in several fish species (Driedger et al. 2010, Dubé et al. 2005, Weber et al. 2008) have been documented as a result of exposure to several different types of MMEs. Because effects can be harmful to the aquatic ecosystems, metal mining operations typically monitor their effluents closely to ensure safe release into the environment.

In Canada, the federal Fisheries Act, through the Environmental Effects Monitoring (EEM) program in the Metal Mine Effects Regulations (MMER), legislates subject metal mines to monitor their effluents and verify that they do not affect aquatic invertebrates, fish, or fisheries (Ribey et al. 2002). If effects are confirmed through the program, investigations of cause (IOC) approaches are subsequently followed. The IOC approaches consist of confirming and identifying the source, and identifying and characterizing the chemical(s) (Hewitt et al. 2003, 2005). Chemical characterization and identification, as the final tier, includes identification of specific causes of any confirmed observed effects. In cases when MMEs are found to cause effects, determining the causes is often complex. The aquatic environments that receive MMEs not only contain high concentrations of a variety of metals and metalloids, but are also often

contaminated by organics/endocrine disrupting compounds from other sources (e.g. waste water treatment plants, urban run-off) (Dubé et al. 2006, Rickwood et al. 2008). In addition, there might be historical contamination from past mining operations or other industries, or multiple effluents from a single mining operation. The different type of MME exposures can lead to different types of effects (Rozon-Ramilo et al. 2011a) or potentially lead to cumulative effects due to the exposure to multiple stressors. Metals, organics, and/or historical contamination can each contribute to effects in the aquatic environment, confounding the central question of determining whether the cause of observed effects is the current effluent under regulation. Consequently, identifying causative chemicals or metals is a main objective if MMEs are found to cause effects in the environment.

Traditionally, laboratory metal toxicology studies have mostly involved assessing effects on fish with exposures to a single metal, often with only a single route of exposure (i.e., waterborne or dietary). Although these studies provide valuable information on metal toxicity and uptake routes, they might not be environmentally realistic in waterbodies exposed to metal mixtures such as MMEs. Multiple metals can cause different effects than single metals, through additive, less than additive, or more than additive relationships (Norwood et al. 2003). In order to assess effects of complex metal effluents, and to include both waterborne and dietary exposures, the use of mesocosms has recently been introduced into monitoring programs (Driessnack et al. 2011, Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a, 2011b). Because dietary uptake is an important source of toxicity with certain metals (e.g., Cu and Se), the addition of a live food source also exposed to the contaminants increases the environmental relevance (Kamunde et al. 2002, Lemly 1997, Muscatello et al. 2006). Trophic transfer of metals and metal bioavailability are believed to play an important role in contributing to effects,

including metal accumulation in fish tissues and altered reproduction in fathead minnows (*Pimephales promelas*) (Driessnack et al. 2011, Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a, 2011b).

It is to be noted that the specific metals that contribute to the biological effects from MME exposures are still largely unknown. Metals such as Cu, Ni, and Se are known to cause reproductive impacts in fish under elevated exposure levels. Elevated Cu and Ni concentrations have each been shown to contribute to lower egg production in minnows (Geckler et al. 1976, Horning and Neiheisel 1979, Mount 1968, Pickering 1974). Se bioaccumulation has been reported to cause larval deformities and reproductive failure in fish when consumed through the diet (Holm et al. 2005, Lemly 1997). Furthermore, Cu and Se uptake through the diet in lakes exposed to MMEs is believed to contribute to decreased reproductive condition in fish (Pyle et al. 2005). Cu, Ni, and Se are commonly found at elevated concentrations in MMEs, however it is necessary to explore whether they are responsible for toxic effects under specific conditions of the MMEs. Metal bioavailability and toxicity is highly influenced by water chemistry parameters, such as pH, alkalinity, and hardness, although each metal is not affected in the same way (Niyogi and Wood 2004). MMEs are often released into the environment under slightly acidic and high hardness conditions, which could influence metal toxicity to resident biota.

The objective of this research was to investigate possible causes of toxicity in adult fathead minnows exposed to a Canadian MME using an approach similar to that followed in IOC (i.e., to identify particular metals responsible for toxic effects). Specifically, this study aimed to compare and contrast fathead minnow response patterns (tissue-specific metal bioaccumulation, and fish morphometrics and reproductive performance) from exposure to a MME relative to effluent equivalent doses of Cu, Ni and Se, both singly as well as in mixture. We hypothesized

that fathead minnows exposed through the water and diet to similar concentrations of these three metals to those found in 45% PWE, under matching water chemistry conditions, would exhibit comparable tissue-specific metal bioaccumulation and alterations in reproductive performance. We examined a MME which has been shown to lead to bioaccumulation of a variety of metals in benthic invertebrate and fish tissues, as well as induce reproductive changes in breeding pairs of fathead minnows in a number of previous studies (Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a, 2011b). This MME - Process Water Effuent (PWE) - is released at volumes of approximately 51,000,000 m^3/year (2009) into the Junction Creek Watershed, near Sudbury, Ontario, Canada. Environmental concentration of PWE has been estimated at 45% in the receiving environment based on discharge and stream conditions (Rickwood et al. 2006a).

2.2 MATERIALS & METHODS

2.2.1 Study site, timeline, and mesocosms

This research was performed at the University of Saskatchewan in Saskatoon, SK, Canada, and consisted of three separate studies. The first study (Cu *vs*. 45% PWE) was performed from March to May 2009, the second study (Ni or Se *vs*. 45% PWE) was performed in December 2009 to February 2010, and the third study (Cu, Ni, and Se mixture *vs*. 45% PWE) was performed from January to March 2011. Each study involved the use of multi-trophic mesocosms with up to 8 replicate, 10.3-L circular polyethylene artificial streams in order to expose breeding pairs of fathead minnows to MME, a single metal, or metal mixture. These mesocosms allow for isolated waterborne exposures, or simultaneous waterborne and dietary exposures over a period of 21 days, as a means of assessing chronic effects of MMEs on both invertebrates and fish. These multi-trophic mesocosms are an accepted component of Environment Canada's EEM program, and have been described in details by Hruska and Dubé

(2004) and Rickwood *et al.* (2006). These mesocosms have been found to be useful in evaluating reproductive effects in fish, and bioaccumulation of metals from MMEs to target organisms/tissues (e.g., *Chironomus dilutus*, fathead minnow liver, gonad, gill, and carcass tissues) in Ontario (Dubé et al. 2006, Hruska and Dubé 2004, 2005, Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a, 2011b), New Brunswick (Dubé et al. 2005), Saskatchewan (Driessnack et al. 2011), and the Northwest Territories (Spencer et al. 2008). Animal use in the present studies was approved by the University of Saskatchewan Committee on Animal Care and Supply (UCACS) and Animal Research Ethics Board (AREB).

2.2.2 Reference water, process water effluent, and metal treatments

In our previous studies with MMEs, Vermillion River water had been the source of reference water (RW) for field-based mesocosms near Sudbury, Ontario, Canada (Rozon-Ramilo et al. 2011a). For the present laboratory study, synthetic RW with water chemistry parameters matched to Vermillion River water conditions was used. The same approach was also performed in a previous laboratory investigation with PWE (Rozon-Ramilo et al. 2011b). Therefore, RW in the present study consisted of a mixture of reverse osmosis (RO) water and dechlorinated laboratory water at concentrations of approximately 65% RO and 35% laboratory water in order to match the hardness, pH, alkalinity, and background metal concentrations of Vermillion River water (Rozon-Ramilo et al. 2011a). This mixture was also used as dilution water for the 45% PWE. The exposure water in single metal and mixed metals treatments were also made up of the same ratio of RO and laboratory, and supplemented with calcium sulphate and sodium chloride under constant stirring, in order to match water hardness, salinity, pH, and alkalinity of the 45% PWE.

Process water effluent (PWE) was shipped weekly from Sudbury, Ontario to the University of Saskatchewan. For these studies, PWE was chosen to be used at a concentration of 45% dilution based on environmentally relevant concentrations in the receiving stream watershed. Concentrations of Cu, Ni, and Se in each of the respective single metal treatments were based on concentrations typically found in 45% PWE. Historic ranges of Cu, Ni, and Se in 45% PWE have been reported between 50-95 µg/L, 53-114 µg/L, and 7-10 µg/L, respectively (Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a). Therefore, the concentrations used for the present studies included Cu at 60 µg/L added as copper chloride dehydrate, Ni at 90 µg/L added as nickel (II) nitrate hexahydrate, and Se at 10 µg/L added as sodium selenate. Selenate was used because previous samples analyzed through ion chromatography-inductively coupled plasma-mass spectrometry indicated that selenate is the major species (~90%) of Se present in the PWE (unpublished data). Due to changes in the mining process from a temporary metal production stoppage, compositions of metals in 45% PWE differed from historic ranges. Thus, concentrations of metals used in the metal mixture treatment (Cu, Ni, and Se) were modified slightly (specifically, lower Cu – 30 µg/L, and higher Ni – 110 µg/L, compared to typical concentrations). Each metal treatment was produced every 1.5 to 2 days in 330-L holding tanks and stored for more than 24 hours prior to use in the experimental exposures in order to achieve chemical equilibrium. This allowed for daily renewal of the metal treatments in the trophic transfer system.

2.2.3 Trophic-transfer system

Chironomus dilutus were obtained from in-house laboratory cultures. Experimental *C. dilutus* cultures were raised in RW, 45% PWE, or one of the metal treatments in order to ensure fathead minnows were exposed to potential contaminants through both the water and diet (see

Tables 2-1, 2-2, and 2-3 for composition). Egg sacs of *C. dilutus* were isolated from laboratory held brood stocks and distributed as 2 egg sacs per stream (repeated every 7 days for 3 weeks) in order to provide adequate food (1 g/day of 3rd and 4th instar *C. dilutus* larvae) to the breeding pairs of fathead minnows for the duration of the 21-day exposure period (Rickwood et al. 2006a). Egg sacs were placed directly into the multi-trophic mesocosms and water temperature was maintained at 23°C (±2°C). Water or treatment exchanges began after the first 4 days of *C. dilutus* culturing and were done at a rate of 1 turnover every 2 days. During the first 4 days of culturing all egg sacs were in water-only in order to reduce variability in culturing success between treatments. Each multi-trophic mesocosm was made up of a sediment layer (~2.5 cm of pre-cleaned silica sand), a feeding barrier to control the amount of *C. dilutus* available to fathead minnows, a spawning tile, and a mesh-screen to prevent adult *C. dilutus* and fathead minnows from escaping the streams. Larval *C. dilutus* were fed a blend of Tetramin^{TM} slurry (~1 g/egg sac) every second day throughout the culturing period and the exposure period.

2.2.4 Pre-exposure period

The pre-exposure period was performed in order to assess baseline fathead minnow reproduction in RW and ensure all breeding pairs met performance criteria for the exposure period. Six to eight-month old fathead minnows were obtained from Osage Catfisheries Inc. (Osage Beach, MO, USA). Body weight, total length, and secondary sex characteristics (banding, nuptial tubercles, dorsal pad, fin dot, and ovipositor) were recorded and each fish was placed randomly into a stream until a male and female were present in each stream. Water exchanges were set at 1 turnover/day and temperature was maintained at 25°C (±2°C) with submersible aquarium heaters under conditions of a 16h light:8h dark photoperiod. Breeding

pairs were fed frozen bloodworms (*C. riparius*) twice daily at a feeding rate of approximately 1 g/day.

Egg production was monitored daily by removing the breeding tile from each stream, scraping the eggs from the tile onto a petri dish, and photographing the eggs. Breeding pairs for the exposure period were selected based on spawning at least once in the pre-exposure period and >80% fertilization success. An egg was considered to be infertile if it was either opaque, had a visibly precipitated yolk, or contained no yolk (Ankley et al. 2001). In order to meet this criteria, the lengths of the pre-exposures varied between studies [14 days for the first study (April 1 to April 15, 2009), 10 days for the second study (January 5 to January 15, 2010) and 7 days for the third study (February 4 to February 11, 2011)]. Eighteen, 28, and 18 pairs were chosen for the exposure period in the first, second, and third study, respectively. Pairs were divided into their respective treatments [RW, 45% PWE, single metal, or metal mixture (MM)] and statistical analyses by one-way ANOVA were performed on the pre-exposure egg production (total number of eggs, eggs/female/day, and breeding attempts) to verify that there were no significant differences between treatments [α=0.05, n=6 (first and third exposures) and n=7 (second exposures)] (see Appendix Figure A-1 for mean daily egg production). Following this analysis, breeding pairs were randomly assigned to a stream within their treatment.

2.2.5 Exposure period

Fathead minnows were exposed to their assigned treatment for 21 days for each study. Water exchanges were again set at 1 turnover/day and temperature was maintained at 25°C (±2°C). Water samples from each treatment were taken on days 7, 14, and 21 in pre-labeled high density polyethylene sample bottles obtained from Testmark Laboratories in Sudbury, Ontario, Canada. Samples were taken directly from a single stream which was chosen randomly from

each mesocosm treatment table, sealed in a ziplock bag, and sent for analysis in a cooler chilled with ice. Weekly water quality measurements were conducted by Testmark Laboratories, in accordance with the analytical methodology of the American Public Health Association (APHA) and US Environmental Protection Agency (EPA). These water samples were analyzed for total metals using inductively coupled plasma-mass spectrometry (ICP-MS) with digestion (concentrations of 41 elements were measured), Ca^{2+} and Mg^{2+} concentrations using ICP-MS without acidification [and converted to total water hardness expressed as the equivalent of calcium carbonate ($CaCO_3$)], anions by Ion Chromatography, and dissolved organic carbon (DOC) and total organic carbon (TOC) with a Dohrman TOC Analyzer. TOC and DOC samples were analyzed using the APHA 5310 standard method. DOC samples were run through a 0.45 µm nylon filter. Daily water quality measurements were performed at the University of Saskatchewan. These measurements included temperature, dissolved oxygen (DO), and conductivity [YSI meter (Yellow Springs Instruments, Yellow Springs, OH, USA)], ammonia (Rolf C. Hagen, Edmonton, AB, Canada), pH (Oakton pHTestr 3, Oakton Instruments, Vernon, IL, USA), and alkalinity (LaMotte Company, Chestertown, MD, USA).

Fathead minnow egg production, egg size, and larval deformities, as well as *C. dilutus* emergence were monitored daily throughout the exposure period. Egg production and fertilization success were determined in the same manner as the pre-exposure period. Egg sizes were determined by randomly selecting 10 eggs per brood and analyzing them with Image Pro Plus 6.1 (Media Cybernetics Inc., Maryland, USA). After photographing the eggs, they were placed into egg cups, returned to the respective treatment, and aerated. Eggs were monitored in the egg cups until all were hatched or killed by fungal infection. Infected eggs were counted and removed daily. After complete hatching, larvae were moved into petri dishes and photographed

again with the microscope. Larval deformities were determined by analyzing photographs for edema or spinal deformities.

At the end of the exposure period, fathead minnows were anesthetized with tricaine methanesulfonate (MS-222), assessed for secondary sex characteristics, total body weight, total length, and dissected to obtain livers, gills, gonads, and the carcass. Weights of these individual tissues were also obtained. Densities of *C. dilutus* were measured on day 21 by sampling three 9 cm^2 cores per stream. Biofilm and *C. dilutus* were collected from each stream and these, along with the female fish tissue samples, were frozen in a cooler of dry ice and sent to Testmark Laboratories for metal analysis. Only female tissues were analyzed for metal concentrations in the present study because past studies have shown that resident female fish exposed to MMEs in the Junction Creek watershed accumulated greater concentrations of metals relative to the male fish (Weber et al. 2008). We were unable to collect sufficient quantities of biofilm for metal analysis in the final study due to low biofilm production on the streams. Metal analysis in the fish tissues and *C. dilutus* were done by ICPMS with Microwave Digestion. Testmark Laboratories used method blanks, positive controls, blank spikes and laboratory duplicates with water samples, as well as method blanks and DOLT-3 dogfish (*Squalus acanthias*) liver reference materials (obtained from the National Research Council of Canada) for the biological samples as their methods of quality control. Percentage recovery for selected elements (Cu, Ni, Se) was within the range of 96.9–113.0% in study 1, 93.5-122.6% in study 2, and 89.7-111.0% in study 3. Percent recovery rates were similar to previously reported values for these types of tissues (Rozon-Ramilo et al. 2011a, 2011b).

2.2.6 Metal speciation

Speciation modeling was performed using Visual MINTEQ, version 3.0 (KTH, Department of Land and Water Resources Engineering, Stockholm, Sweden). To consider metal complexation with organic matter, we assumed that 40% of active DOC was fulvic acid and 60% was humic acid (Kamunde and MacPhail 2011). Model input data were taken directly from the mean weekly water chemistry analyses and therefore included all elements, metals, and ions that were determined to be present in the various treatments.

2.2.7 Exposure analysis

Data were analyzed and graphed using PASW Statistics 18.0.0 (SPSS, Chicago, IL) and Sigmaplot® Version 11 (San Jose, CA, USA). Water chemistry and metal burdens in *C. dilutus* and fish tissues were analyzed using one-way analysis of variance (ANOVAs). Cumulative eggs/female and cumulative total spawning events were analyzed by Kolmogorov-Smirnov tests, while mean adult survival, condition factor, relative egg size, liver somatic indices [LSI (%)], gonadosomatic indices [GSI (%)], mean total deformities (%), mean fertilization success (%), and mean *C. dilutus* densities were also analyzed using one-way ANOVAs. The Shapiro-Wilk test was used to test parametric assumptions for normality and Levine's test was used to test for homogeneity of variance prior to the one-way ANOVAs. Data that failed these assumptions were either transformed (arcsin (%) or $\log_{10}$) or analyzed using the non-parametric Kruskal-Wallis test. If a significant difference was detected by one-way ANOVA ($p \leq 0.05$), Tukey's HSD post-hoc test was then used to determine if differences were present between the treatment and the references, and/or among the treatments.

2.3 RESULTS

2.3.1 Water chemistry and metals

The water chemistry parameters and elevated metal concentrations for each of the three studies are presented in Table 2-1, Table 2-2, Table 2-3. These tables include all water chemistry parameters that were measured during the studies, however only metal concentrations that were statistically different between treatments are presented, with the exception of Cu in study 2 (see Appendix Table A-1 for composition of 45% PWE). Overall, concentrations of over forty metals and elements were measured, including some of the common toxic metals such as Cd, Hg, Pb and Zn. However, concentrations of these metals were either below detection limits or found not to be elevated in the 45% PWE relative to RW in any of the studies (ANOVA; $p>0.05$). Single metal, mixed metals, and 45% PWE treatments were similar in water chemistry parameters in all three studies. Conductivity, hardness, chloride, sulfate, and calcium levels were generally not significantly different between the single metal, mixed metals, or 45% PWE treatments within each of the studies. Nitrate, magnesium, barium, boron, cobalt, lithium, and rubidium were typically elevated in the 45% PWE relative to the other treatments within each study.

2.3.2 Waterborne single metal and mixed metal treatments

In the first study, concentrations of total Cu were significantly increased in the Cu-only and 45% PWE treatments relative to the RW and were not significantly different from each other (Table 2-1). In the second study, concentrations of total Ni and total Se were each significantly elevated in their respective single metal treatments and the 45% PWE relative to the RW (Table 2-2). Concentrations of total Ni were not significantly different between the Ni-only treatment and the 45% PWE treatment, however concentrations of total Se were significantly different in the Se-only treatment compared to the 45% PWE treatment. This was due to lower

Table 2-1 – Water chemistry and total elevated metal concentrations sampled from reference water (RW), 45% process water effluent (PWE), and Cu treatments in the 45% PWE *vs*. Cu study, taken as daily water quality measurements and weekly water samples.

		Units	RW	45%PWE	Cu
General Chemistry	**Temperature**	°C	24.4±0.1[a]	24.7±0.1[a]	24.7±1.2[a]
	pH	pH	7.62±0.02[a]	6.91±0.05[b]	7.47±0.02[c]
	DOC*	mg/L	7.2±1.3[a]	5.1±0.7[a]	6.4±1.1[a]
	Alkalinity	mg/L	40.4±0.6[a]	27.2±1.2[b]	36.7±0.6[c]
	Ammonia	mg/L	0.057±0.015[a]	0.394±0.072[b]	0.329±0.139[ab]
	Conductivity	µS/cm	173.2±3.2[a]	1376.6±26.3[b]	1454.5±13.2[b]
	Dissolved Oxygen	%	85.9±0.7[a]	86.9±0.7[a]	87.4±0.7[a]
	Total Hardness (as $CaCO_3$)	mg/L	47.3±4.7[a]	519.3±32.0[b]	541.0±23.8[b]
	Chloride	mg/L	5.02±1.18[a]	38.73±5.00[b]	63.4±4.70[c]
	Nitrate	mg/L	0.267±0.217[a]	1.837±0.091[b]	0.820±0.291[a]
	Sulfate	mg/L	25.00±3.76[a]	597.67±62.85[b]	575.33±47.21[b]
	Total Calcium	mg/L	11.01±1.40[a]	170.00±17.90[b]	224.33±19.00[b]
	Total Magnesium	mg/L	6.46±0.60[a]	22.20±2.45[b]	6.25±0.67[a]
Metals	**Barium**	µg/L	10.57±0.93[a]	23.83±0.30[b]	22.10±1.21[b]
	Boron	µg/L	20.07±2.71[a]	50.60±3.39[b]	18.33±2.33[a]
	Cobalt	µg/L	0.05±0.00[a]	3.88±0.73[b]	0.34±0.02[c]
	Copper	µg/L	7.03±1.19[a]	80.10±12.53[b]	58.83±8.67[b]
	Lithium	µg/L	3.33±0.83[a]	23.67±0.88[b]	3.50±1.00[a]
	Nickel	µg/L	2.83±0.54[a]	99.87±15.74[b]	10.40±0.64[c]
	Rubidium	µg/L	0.70±0.20[a]	23.73±1.03[b]	0.93±0.22[a]
	Selenium	µg/L	0.50±0.00[a]	7.20±0.31[b]	1.80±0.85[a]

Sample sizes were n=21 for parameters that were measured daily (temperature, pH, alkalinity, ammonia, conductivity, and dissolved oxygen), and n=3 for parameters that were measured weekly (all remaining general chemistry parameters and metals). Concentrations of metals or other elements that were not statistically different among treatments are not shown.
Data are presented as mean ± standard error.
Means that do not share letters are statistically different from one another (one-way ANOVA; Tukey HSD post hoc test, $p<0.05$).
*DOC – dissolved organic carbon

Table 2-2 – Water chemistry and total elevated metal concentrations sampled from reference water (RW), 45% process water effluent (PWE), Ni, and Se treatments in the Ni or Se *vs*. 45% PWE study, taken as daily water quality measurements and weekly water samples.

		Units	RW	45%PWE	Ni	Se
General Chemistry	**Temperature**	°C	24.4±0.1[a]	24.3±0.1[a]	24.7±0.2[a]	24.5±0.3[a]
	pH	pH	7.72±0.02[a]	7.15±0.03[b]	7.52±0.03[c]	7.47±0.04[c]
	DOC*	mg/L	4.6±0.4[a]	3.4±0.8[a]	3.2±0.6[a]	3.5±0.3[a]
	Alkalinity	mg/L	51.5±1.4[a]	25.5±1.0[b]	47.0±1.2[a]	50.3±1.6[a]
	Ammonia	mg/L	0.660±0.152[a]	0.980±0.198[ab]	0.829±0.144[a]	1.542±0.219[b]
	Conductivity	µS/cm	200.1±4.4[a]	1505.9±5.0[b]	1407.9±7.9[c]	1397.9±10.2[c]
	Dissolved Oxygen	%	79.7±1.4[a]	82.4±0.8[a]	80.4±1.4[a]	78.5±1.6[a]
	Total Hardness (as $CaCO_3$)	mg/L	52.6±9.5[a]	692.3±16.4[b]	616.7±30.6[bc]	578.3±17.7[c]
	Chloride	mg/L	3.89±0.54[a]	31.70±1.35[b]	54.37±4.75[c]	56.00±2.97[c]
	Nitrate	mg/L	0.110±0.032[a]	0.277±0.050[b]	0.100±0.000[a]	0.100±0.000[a]
	Sulfate	mg/L	26.40±3.75[a]	587.33±38.75[b]	443.67±49.85[c]	467.33±39.18[bc]
	Total Calcium	mg/L	8.75±1.74[a]	232.67±9.68[b]	229.33±26.17[b]	249.33±1.33[b]
	Total Magnesium	mg/L	7.87±1.44[a]	47.50±2.12[b]	7.18±1.30[a]	8.55±0.83[a]
Metals	**Barium**	µg/L	10.30±1.25[a]	26.13±1.04[b]	14.67±2.46[a]	13.90±0.83[a]
	Boron	µg/L	22.27±5.46[a]	64.93±2.54[b]	21.37±3.40[a]	24.07±1.85[a]
	Cobalt	µg/L	0.05±0.00[a]	1.58±0.28[b]	0.45±0.08[a]	0.48±0.07[a]
	Copper	µg/L	9.60±0.40[a]	12.83±2.00[a]	7.97±1.42[a]	8.27±0.17[a]
	Lithium	µg/L	4.63±1.07[a]	41.67±1.20[b]	4.03±1.53[a]	5.33±1.42[a]
	Manganese	µg/L	1.30±0.06[a]	15.57±1.60[b]	2.45±1.12[a]	2.27±1.10[a]
	Nickel	µg/L	3.80±2.70[a]	120.20±20.47[b]	91.33±18.17[b]	12.40±0.25[c]
	Rubidium	µg/L	0.50±0.00[a]	26.67±0.64[b]	0.50±0.00[a]	0.50±0.00[a]
	Selenium	µg/L	0.50±0.00[a]	4.17±0.49[b]	0.50±0.00[a]	10.30±1.64[c]

Sample sizes were n=21 for parameters that were measured daily (temperature, pH, alkalinity, ammonia, conductivity, and dissolved oxygen), and n=3 for parameters that were measured weekly (all remaining general chemistry parameters and metals). Concentrations of metals or other elements that were not statistically different among treatments are not shown.
Data are presented as mean ± standard error.
Means that do not share letters are statistically different from one another (one-way ANOVA; Tukey HSD post hoc test, $p<0.05$).
*DOC – dissolved organic carbon

concentrations of Se in the 45% PWE than what is typically observed. Concentrations of total Cu, Ni, and Se in the mixed metals study (study 3) were significantly different between the mixed metal and 45% PWE treatments (Table 2-3) but were similar to levels from the first two studies.

2.3.3 Copper, nickel, and selenium speciation in exposure water

Metal speciation modeling results suggested similar speciation patterns between 45% PWE, Cu, Ni, Se, and the mixed metals treatments. The free ion Cu^{2+} was present at 5.44% (study1), 1.00% (study 2), and 2.24% (study 3) in the 45% PWE treatments. In the Cu treatment from study 1, Cu^{2+} was present at 0.92%, while during the MM treatment it was present at 0.65%. Ni^{2+} was present at 53.55% (study1), 55.80% (study 2), and 53.74% (study 3) in the 45% PWE treatments. In the Ni treatment from study 2, Ni^{2+} was present at 56.56%, while during the MM treatment it was present at 51.74%. Selenium was primarily in the selenate form, as found during the preliminary trials (unpublished data).

2.3.4 Metal concentrations in biofilm, Chironomus dilutus, and fish tissues

2.3.4.1 Copper

Concentrations of Cu were significantly increased in the biofilm and *C. dilutus* tissues in each treatment with elevated waterborne Cu (Cu, 45% PWE, and MM) relative to the RW treatments (Figure 2-1a & Figure 2-1b). In study 1, there was accumulation of Cu in the liver tissues of fathead minnows in both the Cu-only and 45% PWE treatments. This pattern, however, was not observed in the mixed metal exposure. Minor increases in concentrations of Cu were observed in the ovaries and gills of the MM treatment relative to the reference, but not in the gills of the 45% PWE treatment relative to the MM treatment or the 45% PWE treatment relative to the RW treatment in study 3.

Table 2-3 – Water chemistry and total elevated metal concentrations sampled from reference water (RW), 45% process water effluent (PWE), and metal mixture (MM) treatments in the MM *vs.* 45% PWE study, taken as daily water quality measurements and weekly water samples.

		Units	RW	45%PWE	MM
General Chemistry	**Temperature**	°C	23.9±0.0[a]	24.4±0.2[a]	23.9±0.2[a]
	pH	pH	7.71±0.03[a]	7.19±0.09[b]	7.61±0.03[a]
	DOC*	mg/L	4.0±0.2[ab]	5.0±0.3[a]	3.7±0.2[b]
	Alkalinity	mg/L	32.2±0.8[a]	23.6±2.5[b]	37.7±1.9[a]
	Ammonia	mg/L	0.165±0.100[a]	2.70±0.511[b]	1.317±0.300[a]
	Conductivity	µS/cm	146.6±2.6[a]	1590.0±16.8[b]	1426.5±13.7[c]
	Dissolved Oxygen	%	85.1±0.7[a]	84.1±0.8[a]	86.2±0.4[a]
	Total Hardness (as $CaCO_3$)	mg/L	49.6±6.3[a]	700.3±23.2[b]	599.7±37.3[b]
	Chloride	mg/L	3.07±0.31[a]	37.87±0.41[b]	70.90±1.65[c]
	Nitrate	mg/L	0.453±0.331	0.500±0.000	0.147±0.047
	Sulfate	mg/L	25.03±4.91[a]	663.00±44.84[b]	707.00±8.39[b]
	Total Calcium	mg/L	12.85±2.53[a]	237.33±10.81[b]	233.67±14.24[b]
	Total Magnesium	mg/L	4.26±0.14[a]	26.13±1.34[b]	3.80±0.38[a]
Metals	**Barium**	µg/L	7.73±0.41[a]	29.93±1.30[b]	12.43±1.60[a]
	Boron	µg/L	23.73±0.78[a]	69.30±1.17[b]	24.10±2.10[a]
	Cobalt	µg/L	0.05±0.00[a]	3.45±0.76[b]	0.44±0.07[c]
	Copper	µg/L	11.20±1.87[a]	56.87±3.79[b]	33.57±2.32[c]
	Lithium	µg/L	4.43±0.99[a]	30.67±1.76[b]	3.63±1.13[a]
	Molybdenum	µg/L	0.50±0.00[a]	3.67±0.38[b]	0.73±0.23[a]
	Nickel	µg/L	2.53±0.33[a]	77.33±5.88[b]	105.97±8.05[c]
	Rubidium	µg/L	0.50±0.00[a]	31.37±1.35[b]	0.70±0.20[a]
	Selenium	µg/L	0.50±0.00[a]	7.27±0.48[b]	10.87±0.82[c]

Sample sizes were n=21 for parameters that were measured daily (temperature, pH, alkalinity, ammonia, conductivity, and dissolved oxygen), and n=3 for parameters that were measured weekly (all remaining general chemistry parameters and metals). Concentrations of metals or other elements that were not statistically different among treatments are not shown.

Data are presented as mean ± standard error.

Means that do not share letters are statistically different from one another (one-way ANOVA; Tukey HSD post hoc test, $p<0.05$).

*DOC – dissolved organic carbon

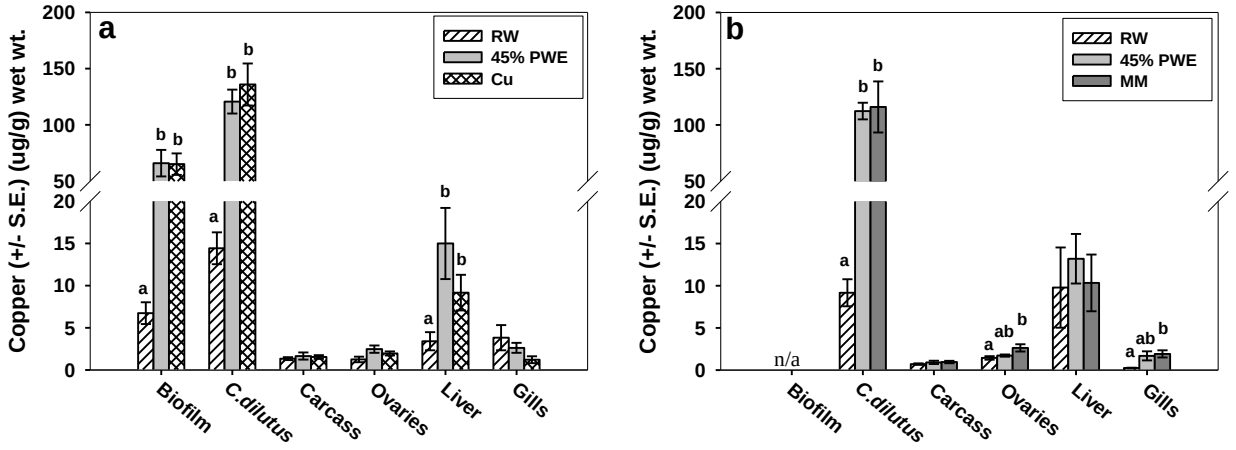

Figure 2-1 - Copper concentrations (mean ± S.E.) in biofilm, *C. dilutus*, and fathead minnow (*P. promelas*) tissues from the Cu *vs*. 45% process water effluent (PWE) study (a) and the metal mixture (MM) *vs*. 45% PWE study (b) at the end of the 21-day exposure period. Within each tissue, means that do not share letters are statistically different from one another (one-way ANOVA; Tukey HSD post hoc test, $p<0.05$). Biofilm was not available (n/a) in sufficient quantities to be collected during the MM study.

2.3.4.2 Nickel

Concentrations of Ni were also significantly increased in the biofilm and *C. dilutus* of each treatment with elevated waterborne Ni (Ni, 45% PWE, and MM) relative to the RW treatments (Figure 2-2a & Figure 2-2b). Bioaccumulation of Ni in fathead minnow tissues was generally low. Small increases in concentrations of Ni were observed in the ovaries of 45% PWE and Ni-only treatments relative to the Se treatment in study 2, and the ovaries of the MM treatment relative to RW and 45% PWE treatments in study 3. There were also elevated concentrations of Ni in the liver during study 3 in the MM treatment relative to the RW and 45% PWE treatments. Bioaccumulation of Ni was observed in the gills of the 45% PWE and MM treatments relative to the RW treatment in study 3, however these concentrations were lower than concentrations measured in gill tissues in any of the treatments during study 2.

2.3.4.3 Selenium

Concentrations of Se were significantly elevated in the biofilm of the Se-only treatment compared to all other treatments (Figure 2-3a). Concentrations of Se were elevated in the *C. dilutus* of Se-only, 45% PWE, and MM treatments relative to the reference treatments (Figure 2-3a & Figure 2-3b). The ovaries and livers tended to accumulate Se. Concentrations of Se were significantly elevated in the ovaries of 45% PWE and the Se-only treatments in study 2 (although also significantly different from one another), and in the ovaries of 45% PWE treatment in study 3. Concentrations of Se were also significantly elevated in the livers of 45% PWE and Se-only treatments relative to the RW treatment in study 2, and in the livers of 45% PWE from study 3. No bioaccumulation of Se was observed in the MM treatment fathead minnow tissues in study 3.

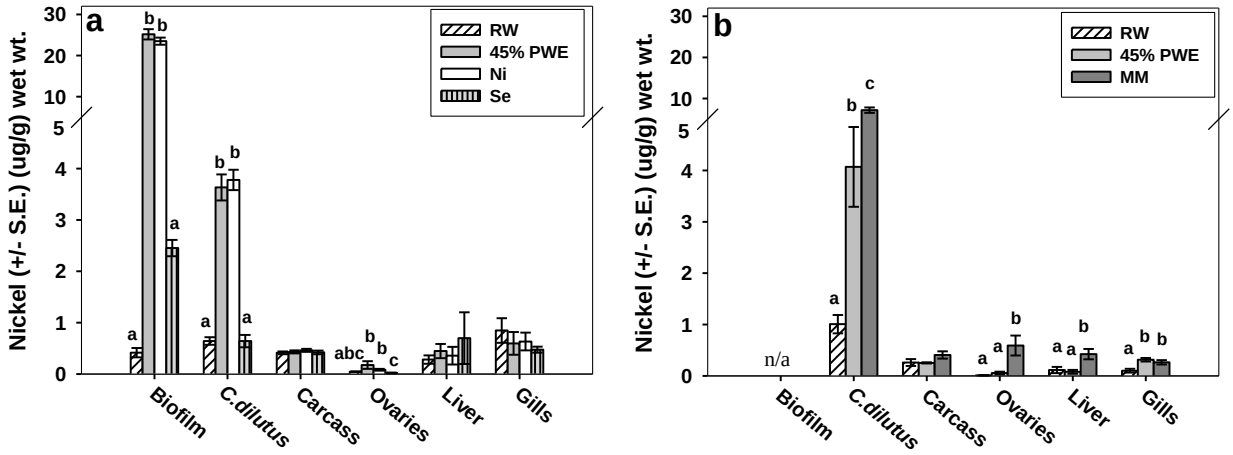

Figure 2-2 – Nickel concentrations (mean ± S.E.) in biofilm, *C. dilutus*, and fathead minnow (*P. promelas*) tissues from the Se or Ni *vs*. 45% process water effluent (PWE) study (a) and the metal mixture (MM) *vs*. 45% PWE study (b) at the end of the 21-day exposure period. Within each tissue, means that do not share letters are statistically different from one another (one-way ANOVA; Tukey HSD post hoc test, $p<0.05$). Biofilm was not available (n/a) in sufficient quantities to be collected during the MM study.

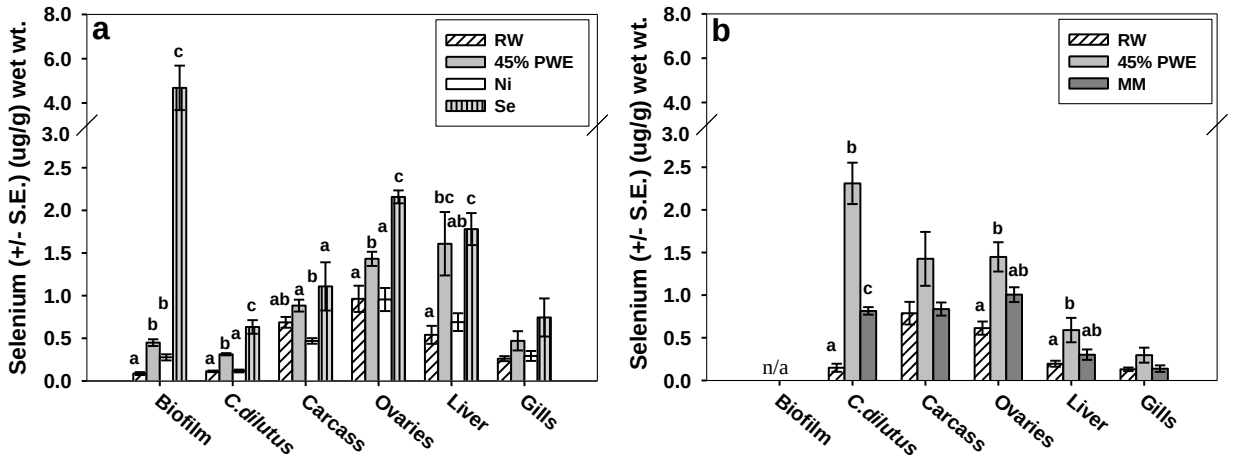

Figure 2-3 – Selenium concentrations (mean ± S.E.) in biofilm, *C. dilutus*, and fathead minnow (*P. promelas*) tissues from the Ni or Se *vs.* 45% process water effluent (PWE) study (a) and the metal mixture (MM) *vs.* 45% PWE study (b) at the end of the 21-day exposure period. Within each tissue, means that do not share letters are statistically different from one another (one-way ANOVA; Tukey HSD post hoc test, $p<0.05$). Biofilm was not available (n/a) in sufficient quantities to be collected during the MM study.

2.3.5 Reproduction

2.3.5.1 Study 1 – copper *vs.* PWE

Although there were some differences in egg production among treatments, there were no statistically significant differences in cumulative mean egg production or cumulative spawning events between any of the treatments in study 1 (Figure 2-4a & Figure 2-4b).

2.3.5.2 Study 2 – nickel or selenium *vs.* PWE

In the second study, there was a statistically significant decrease in cumulative mean egg production in the 45% PWE treatment relative to the RW, Ni-only, and Se-only treatments, small but non-significant change in the egg production of the Ni-only exposed fathead minnows relative to the RW fathead minnows, and no significant difference in the egg production of Se-only exposed fathead minnows relative to the RW fathead minnows (Figure 2-5a). Cumulative total spawning events showed a similar trend to cumulative mean egg production. Fathead minnows exposed to 45% PWE showed a decrease in cumulative total spawning events relative to the RW, Ni-only, and Se-only fathead minnows (Figure 2-5b). There were no significant differences between Ni-only and Se-only fathead minnows compared to the RW fathead minnows, however there was a significant difference between both single metal treatments with Ni-only fathead minnows at a rate of nearly 50% fewer spawning events than Se-only fathead minnows.

2.3.5.3 Study 3 – copper, nickel, and selenium mixture *vs*. PWE

There was a significant decrease in cumulative mean egg production for the 45% PWE exposed fathead minnows in study 3 relative to the RW and MM fathead minnows, however no change in the MM exposed fathead minnows relative to the RW fathead minnows occurred (Figure 2-6a). There was also a decrease in cumulative total spawning events in the 45% PWE

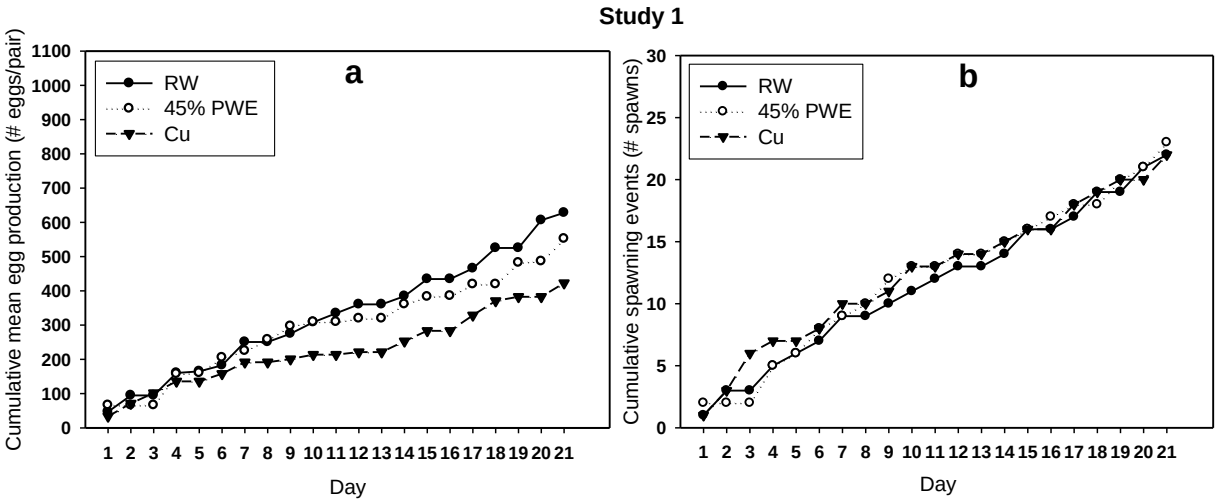

Figure 2-4 – Cumulative daily mean egg production (a) and cumulative daily spawning events (b) for breeding pairs of fathead minnows (*P. promelas*) in the Cu *vs*. 45% process water effluent (PWE) study. There were no statistically significant differences among treatments (two-sample Kolmogorov-Smirnov test; $p>0.05$ for all pairwise comparisons).

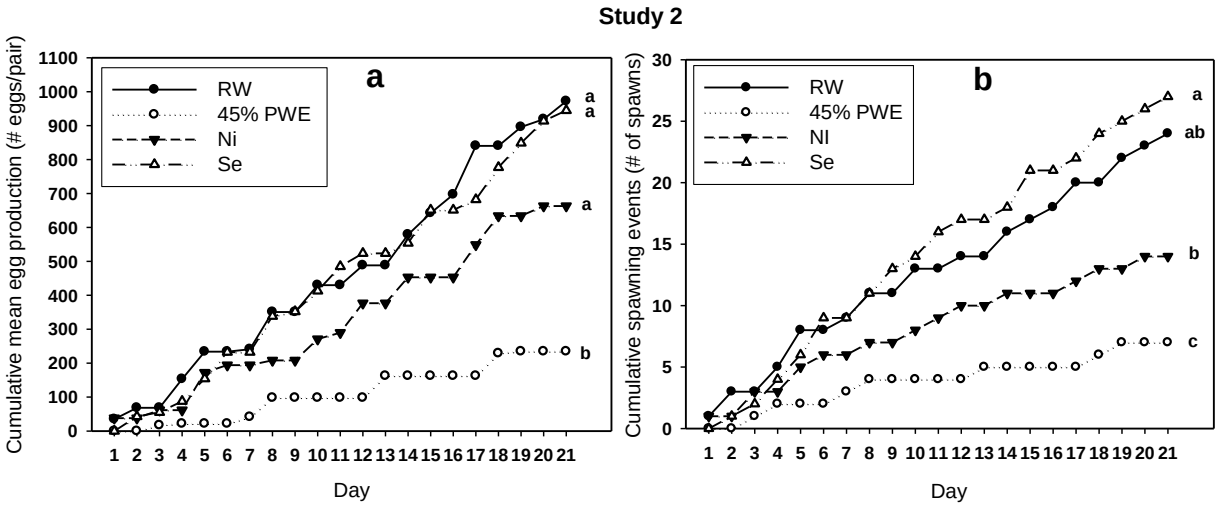

Figure 2-5 – Cumulative daily mean egg production (a) and cumulative daily spawning events (b) for breeding pairs of fathead minnows (*P. promelas*) in the Ni or Se *vs*. 45% process water effluent (PWE) study. Means that do not share letters are statistically different from one another (two-sample Kolmogorov-Smirnov test; $p<0.05$ for all pairwise comparisons).

relative to the RW and MM fathead minnows (Figure 2-6b). There was no significant decrease in spawning events between the RW and MM fathead minnows in this study.

2.3.6 Biological endpoints

Mean adult survival (%) ranged from 71.5% to 100% per treatment, and was not statistically different between any of the treatments within any of the three studies (Kruskal-Wallis, p=0.783, p=0.919, and p=0.586). There were also no statistically significant differences in LSI, GSI, or condition factor (K) in males or females among treatments within any of the three studies (ANOVA; p>0.05) (Table 2-4 and Table 2-5). Rates of larval deformities ranged from 1.7% to 27.8% and primarily consisted of edema. However, no statistically significant differences in larval deformities among treatments were observed in any of the three studies (ANOVA; p>0.05) (see Appendix Figure A-2). There were also no statistically significant differences in hatch rates of eggs among treatments in any of the three studies (ANOVA; p>0.05) (see Appendix Figure A-3).

There was a statistically significant difference in the egg sizes of fathead minnows exposed to 45% PWE in the first study relative to the RW eggs (-7.7%) (Tukey's HSD post hoc test p=0.027), with no significant difference in egg size between the 45% PWE and eggs from the Cu -only treatment fathead minnows (Tukey's HSD post hoc test p=0.173), nor the Cu -only fathead minnows relative to the RW eggs (Tukey's HSD post hoc test p=0.536). There was a trend towards smaller egg sizes in the 45% PWE, Ni-only, and Se-only treatments, and the 45% PWE and MM from study 2 and 3, however these trends were each not statistically significant (ANOVA, p=0.432 and p=0.079, respectively) (see Appendix Figure A-4).

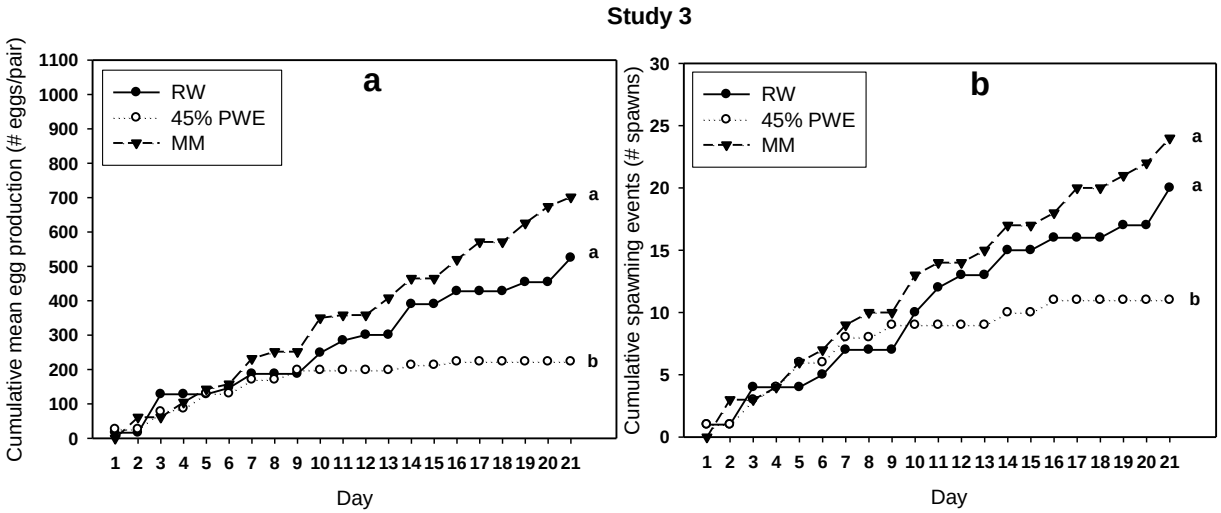

Figure 2-6 – Cumulative mean egg production (a) and cumulative spawning events (b) for breeding pairs of fathead minnows (*P. promelas*) in the metal mixtures (MM) *vs.* 45% process water effluent (PWE) study. Means that do not share letters are statistically different from one another (two-sample Kolmogorov-Smirnov test; $p<0.05$ for all pairwise comparisons).

Table 2-4 – Gonadosomatic index (GSI), liver somatic index (LSI), and condition factor (K) for female fathead minnows (*P. promelas*) from the Cu *vs*. 45% process water effluent (PWE), Ni or Se *vs*. 45% PWE, and metal mixture (MM) *vs*. 45% PWE studies

		N	GSI (%)	LSI (%)	K
Study 1	**RW**	6	17.8±4.9	5.1±0.4	1.0±0.1
	45% PWE	6	13.2±1.3	5.2±0.1	1.1±0.0
	Cu	6	16.8±2.4	4.9±0.5	1.2±0.1
Study 2	**RW**	6	15.9±1.2	3.2±0.8	1.2±0.0
	45% PWE	6	17.4±1.7	4.0±0.6	1.2±0.1
	Ni	5	19.8±2.7	4.3±0.6	1.2±0.1
	Se	6	18.2±2.2	4.4±0.3	1.3±0.1
Study 3	**RW**	5	16.2±1.8	2.6±0.4	1.1±0.0
	45% PWE	6	10.5±2.2	2.5±0.3	1.0±0.0
	MM	6	12.3±2.2	3.0±0.2	1.0±0.0

Data are presented as mean ± S.E.

Table 2-5 – Gonadosomatic index (GSI), liver somatic index (LSI), and condition factor (K) for male fathead minnows (*P. promelas*) from the Cu *vs*. 45% process water effluent (PWE), Ni or Se *vs*. 45% PWE, and metal mixture (MM) *vs*. 45% PWE studies

		N	GSI (%)	LSI (%)	K
Study 1	**RW**	6	1.8±0.2	2.9±0.5	1.1±0.0
	45% PWE	6	1.3±0.2	3.4±0.3	1.1±0.1
	Cu	5	1.2±0.2	2.6±0.5	1.1±0.1
Study 2	**RW**	5	1.7±0.1	3.9±0.3	1.2±0.0
	45% PWE	6	1.1±0.1	3.0±0.4	1.2±0.0
	Ni	7	1.3±0.2	2.8±0.2	1.1±0.0
	Se	5	1.3±0.1	3.4±0.2	1.1±0.1
Study 3	**RW**	5	0.8±0.2	2.2±0.2	1.1±0.0
	45% PWE	5	1.1±0.1	1.7±0.3	1.1±0.1
	MM	6	0.9±0.1	1.7±0.2	1.2±0.0

Data are presented as mean ±S.E.

2.3.7 Densities of Chironomus dilutus

There were no significant differences in densities of *C. dilutus* larvae among treatments at the end of the 21 day exposure period during the first study (ANOVA; p=0.807) (Figure 2-7). There was a trend towards lower densities of *C. dilutus* larvae in the 45% PWE treatment relative to the other treatments in the second study (0.5 larvae per cm^2 fewer in 45% PWE relative to the other treatments), however this difference was not statistically significant (p=0.264). In study 3, the 45% PWE treatment had significantly fewer *C. dilutus* larvae on day 21 than the RW treatment (Tukey's HSD post hoc test p=0.004).

2.4 DISCUSSION

2.4.1 Conditions of the reference water (RW)

It should be noted that Cu concentrations ranged from 7.03 - 11.20 µg/L in the RW used in the three present studies. Concentrations of Cu in water above 2 - 4 µg/L, depending on the water hardness, are considered elevated as per the Canadian Water Quality Guidelines for the Protection of Aquatic Life (CCME 2008). However, a mean Cu concentration of approximately 9.0 µg/L was recorded in the RW obtained from the Vermillion River during a field-based mesocosm study (Rozon-Ramilo et al. 2011a), which suggests that the Cu concentration in the RW used in present studies was similar to background Cu concentrations in the field and environmentally realistic. Concentrations of Cu in multi-trophic systems performed in the laboratory at the University of Saskatchewan have typically been in the range of 2.10 µg/L (Rozon-Ramilo et al. 2011b) to 9.31 µg/L (Rickwood et al. 2006a), as well. Furthermore, the free ion concentrations of Cu under the existing RW conditions is expected to be low (<0.01%) as derived by geochemical speciation model Visual MINTEQ, version 3.0 (KTH, Department of

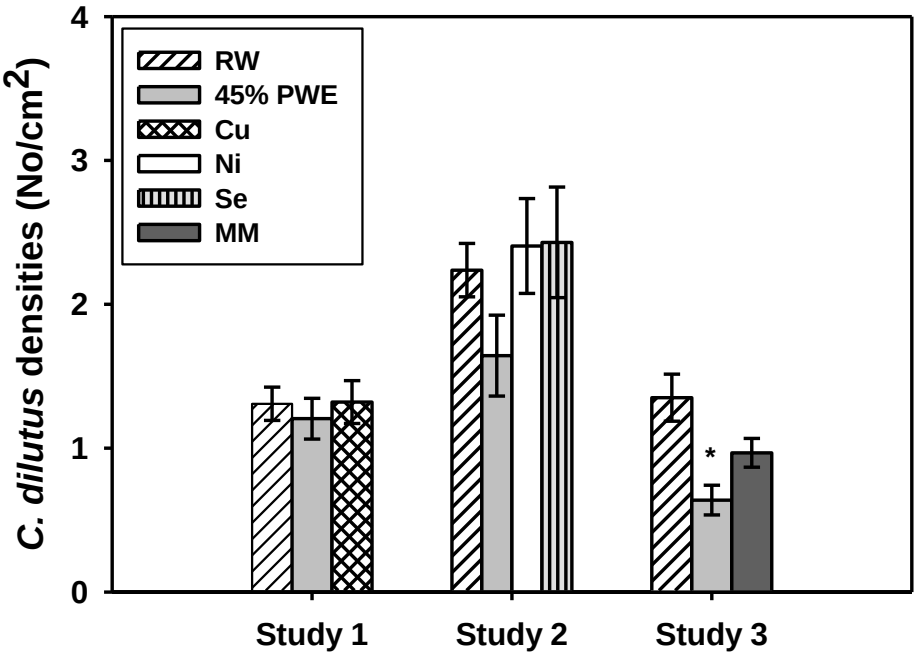

Figure 2-7 - *C. dilutus* densities (No/cm^2) measured on day 21 of the exposure period in each of the treatments from each of the three studies. Treatments were reference water (RW), 45% process water effluent (PWE), Cu, Ni, Se, or Metal Mixture (MM). Optimal densities are considered 3/cm^2 (or 1 g/pair of fathead minnows per day). The asterisk indicates a significant difference from RW (one-way ANOVA; Tukey HSD post hoc test, p=0.004).

Land and Water Resources Engineering, Stockholm, Sweden), suggesting Cu is not readily bioavailable in the water.

The present studies also had relatively high levels of DOC in the RW, which ranged from 4.0 - 7.2 mg/L. DOC concentrations in our studies were a result of the conditions of the multi-trophic mesocosms, which contained live *C. dilutus* larvae, dead *C. dilutus* adults, biofilm, and uneaten Tetramin™. It has previously been suggested that *C. dilutus*, biofilm, and Tetramin™ could be potential sources of organic matter and carbon (Rozon-Ramilo et al. 2011b), and we suspect this to be the case in the present studies as well. The average DOC concentration in lakes near Sudbury, ON has been reported to be approximately 3.7 (±3.7) mg/L (Valois et al. 2011), which is quite similar to the DOC concentrations of our laboratory RW.

2.4.2 Variability in metal concentrations and water chemistry

Overall, concentrations of Cu, Ni, and Se in our single metal, mixed metal and 45% PWE treatments were variable within and among studies. Concentrations of Cu, Ni, and Se in the 45% PWE ranged from 12.83 µg/L to 80.10 µg/L, 77.33 µg/L to 120.20 µg/L, and 4.17 µg/L to 7.27 µg/L, respectively. Typically, metal concentrations in PWE varies over time (see Dubé et al. 2006, Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a, 2011b for values from 2001-2011). PWE composition from all metal mines depends on the types of ores being mined, rates of metal extraction, processing and water recycling, effluent treatment, as well as water quality (Clarke 1974). A temporary shutdown at the mining site in the present study during the Ni and Se single metal exposures resulted in unusually low concentrations of several metals in 45% PWE, particularly Cu and Se. Other water chemistry and quality parameters showed marginal variation among 45% PWE treatments from different studies but were not affected by the mining

shutdown. This variability in metal concentrations and water chemistry could contribute to some differences in effects among treatments and is discussed below.

2.4.3 Reproductive impacts

Previously, one of the most consistent changes observed in fathead minnows exposed to 45% PWE has been a decrease in cumulative egg production and spawning events. As reported in Rozon-Ramilo (2011), four out of the five previous 45% PWE exposure studies observed these types of decreases. Other MME studies have found increases in egg production from uranium mine effluent (Driessnack et al. 2011) and surface water effluent (Rozon-Ramilo et al. 2011a). Previous studies have also found decreases in egg sizes from 45% PWE exposures (Rozon-Ramilo et al. 2011a, 2011b). As a result, there is strong evidence that fathead minnow egg production is a sensitive endpoint to certain MMEs, and these MMEs may have either a stimulatory or inhibitory effect.

Decreases in cumulative mean egg production were observed in fathead minnows exposed to 45% PWE in two of the three studies (80% decrease in study 2 and 60% decrease in study 3, relative to the respective RW exposures). Fathead minnows exposed to single metal or mixed metal treatments did not suffer from any significant reproductive effects, except a moderate difference in egg production between the Ni and RW treatments in study 2 (35% decrease in Ni treatment). However, cumulative mean egg production in the RW was also two-fold higher than that observed in the other two studies (study 1 and study 3), and as a result, it is possible that the difference in egg production between Ni and RW in study 2 is an artifact of above normal RW breeding. In past studies, decreases in egg production and egg sizes resulting from exposure to Cu or Ni (Geckler et al. 1976, Horning and Neiheisel 1979, Pickering 1974), and reduced swelling in fish eggs from Cu exposure have been observed (Jezierska et al. 2009). Selenium has

also contributed to increased larval deformities in fish (Driessnack et al. 2011, Lemly 1997, Muscatello et al. 2006). Overall, metal toxicity is highly related to water chemistry and bioaccumulation. Therefore, toxic effects, such as changes to reproductive output, are likely to be very study-specific.

2.4.4 Waterborne exposures and water chemistry

Water hardness is likely an important factor in regulating metal bioavailability and toxicity in MMEs. The major hardness cations, calcium and magnesium, compete with free metal ions in the water for binding to the uptake sites on the fish gill, and thereby reduce metal accumulation and toxicity (Niyogi et al. 2008, Pyle et al. 2002). The high hardness levels in our single metal, mixed metal, and 45% PWE treatments suggest that bioavailability of metals that compete with calcium and magnesium (e.g., Cu^{2+}, Ni^{2+}) would be relatively low to *C. dilutus* or fathead minnows due to increased competition. Other water chemistry parameters that differed between treatments, such as pH, alkalinity, and DOC, are also known to affect metal bioavailability and toxicity by complexation of free metal ions, and thus, could contribute to observed differences in reproductive effects in fish among treatments. However, our results suggest that the various water chemistry parameters were not major factors in contributing to differences in metal bioaccumulation and toxicity in fathead minnows.

Increases in pH, alkalinity, and DOC are known to decrease the free ion concentrations and bioavailability of waterborne metals by complexation during acute exposures, which therefore reduce the toxicity of the metals (Allen and Hansen 1996, Campbell and Stokes 1985, Meador 1991, Niyogi and Wood 2004, Pyle et al. 2002). For example, metals may bind to DOC and become less bioavailable relative to the conditions of lower DOC, or metal complexes may dissociate under conditions of low alkalinity and pH and become available in their most

bioavailable free ion form (e.g., Cu^{2+} and Ni^{2+}). Free ion concentrations and bioavailability tend to be more closely linked with metal toxicity than total dissolved metal concentrations, particularly when considering waterborne exposures (Vijver et al. 2004). In 45% PWE, pH has been observed to be as low as 6.69±0.43 (Rozon-Ramilo et al. 2011a) and as high as 7.8±0.1 (Hruska and Dubé 2005). This would suggest that MMEs under more acidic conditions are likely to result in increased toxicity. Interestingly, we did not observe a correlation between pH and reproductive toxicity. The 45% PWE from our first study had a consistently lower pH than the 45% PWE from either of our other two studies, but did not contribute to reduced egg production. It could also be expected that fathead minnow egg production would be lower in 45% PWE treatments with low DOC due to higher metal bioavailability. However, based on results from the present studies, it seems unlikely that there is any relationship between lower DOC and decreased egg production. For example, at DOC concentrations of approximately 5 mg/L in 45% PWE, fathead minnows produced fewer eggs than the RW treatment in one exposure (study 3) yet produced a similar number of eggs relative to the RW treatment in another exposure (study 1).

There were some differences in pH, alkalinity, DOC, nitrates, and magnesium concentrations between the 45% PWE, single metal, and mixed metal treatments in our studies. These factors can influence metal toxicity, primarily by influencing metal speciation. However, the metal speciation results suggest similar species of Cu, Ni, and Se among all treatments. Cu was generally not in the free ionic form (Cu^{2+}). The concentration of Cu^{2+} in the exposure water was estimated to be ~5% or less of total copper in each treatment. Selenium existed primarily as selenate (which has been confirmed in the 45% PWE - unpublished data), which is much less toxic relative to the other common inorganic form of selenium, selenite (Brix et al. 2001). These

findings also suggest that the dietary pathway was a more important uptake route for copper and selenium in fish relative to the gills.

The free Ni^{2+}, conversely, was found to be the most common form of nickel in our studies (~50%), therefore its uptake through the gills may have been important. Toxicity in fish due to exposure to Ni is primarily observed through the gills and ultimately affects respiration. Typically, high concentrations of dissolved Ni are required in order to see acute effects. Bioaccumulation of Ni in the gills has been shown to cause damage to gills and cells involved in oxygen exchange at levels in acute tests ranging from 9.7 mg/L to 64 mg/L dissolved Ni (Nath and Kumar 1989, Pane et al. 2004a). These types of studies are generally done at high Ni concentrations, approximately 100-fold higher than levels found in the 45% PWE or our metal treatments and are not environmentally realistic. Chronic Ni exposures at environmentally realistic levels of 243-394 µg/L over longer time frames (40-90 days) have also been shown to cause decreases in gas exchange and respiratory impacts in rainbow trout, although these effects were recorded at a much lower water hardness levels (~140 mg/L $CaCO_3$) (Pane et al. 2004b) relative to that in PWE, nickel-only, or metal-mixture treatments in our studies.

2.4.5 Metal bioaccumulation

Although free ion concentrations in the water may be an important source of toxicity, gill-metal binding and bioaccumulation are believed to better predict toxicity when compared to waterborne Cu^{2+} and Ni^{2+} concentrations, particularly in fish species such as rainbow trout and fathead minnows (Meyer et al. 1999, Pane et al. 2003, 2004a). Furthermore, tissue-specific metal accumulation provides a good indication of the routes of exposure and toxicity. Waterborne exposure is likely to lead to increased metal accumulation in the gills, whereas

dietary uptake is likely to lead to increased metal accumulation mainly in the liver and insignificant or marginal increase in the gills.

Pane *et al.* (2004b) reported concentrations of approximately 3 µg/g in the gills of rainbow trout following 42 days of waterborne exposure to 384 µg/L at a moderate hardness (~140 mg/L $CaCO_3$) in a study designed to examine the effects of Ni on gill functions. The levels of Ni bioaccumulation in the gills in our studies were low at approximately 1 µg/g suggesting that respiratory effects were probably not an issue. Pane *et al.* (2004b) also reported that Ni bioaccumulation occurred primarily in the gills, plasma and kidneys, with no increases in muscles or livers. Our results showed a similar trend, with little bioaccumulation of Ni occurring in the carcass or livers of fathead minnows despite increases in the biofilm and *C. dilutus*, which suggests that dietary uptake of Ni was not significant. Therefore, our results indicate that it is unlikely that Ni bioaccumulation is contributing to toxic effects in fish observed in our studies.

Similarly to Ni, our studies frequently resulted in bioaccumulation of Cu, and Se in biofilm, and bioaccumulation of Cu in *C. dilutus* from single metal, mixed metals, and 45% PWE exposures. These metals have also been found to increase consistently in the biofilm and *C. dilutus* of 45% PWE treatments in past studies (Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a). Despite these consistent increases, Cu and Se did not necessarily result in trophic transfer into the fish tissues.

In fish, liver is the primary organ involved in bioaccumulation and homeostasis of Cu, therefore we would expect any significant increases in Cu to occur in this type of tissue (Bury et al. 2003). However, Cu is a highly regulated essential metal and concentrations in fish have been suggested to vary between 7 and 50 µg/g dry weight (d.w.) in the liver under conditions that would not cause adverse effects to fish [or approximately 1.75-12.5 µg/g wet weight (w.w.),

assuming water content in the liver of fish is approximately 0.75 g of water per gram of wet liver] (Couture and Rajotte 2003). Concentrations of Cu in fathead minnow livers from our studies did not exceed 20 µg/g w.w. and were generally similar among treatments. Concentrations of Cu of 20 µg/g w.w suggest that Cu could be reaching concentrations great enough to cause effects. However, our present studies did not find any significant reproductive or morphometric effects in fathead minnows during exposure to Cu. Other studies have reported greater concentrations of Cu in livers of yellow perch (*Perca flavescens*), ranging from ~27 – 62 µg/g w.w. (Brodeur et al. 1997, Sherwood et al. 2000), which suggests that the concentrations of Cu in the livers of fathead minnows are on the lower range of Cu contamination that could cause energetic effects or stress responses in fish.

Bioaccumulation of Se, like Cu, is known to occur in the livers of fish. To prevent reproductive impairment, 12 µg/g d.w. concentrations of Se have been suggested as a maximum threshold in liver tissues (Lemly 1993). The maximum concentration of Se bioaccumulation that was observed in our studies was 2.67 µg/g d.w. (again, assuming water content in the liver is approximately 75%). Bioaccumulation of Se in the ovaries and eggs of fish is also quite common, however to observe impacts, accumulation levels have been suggested to be at least 17 µg/g d.w. in cutthroat trout and greater than 20 µg/g d.w. in fathead minnows (Janz et al. 2010). We did not measure Se concentrations in eggs, however levels higher than 2.5 µg/g d.w. were not observed in fathead minnow ovaries from any metal, mixed metal, or MME exposure in our study. As a result, it is not surprising that we did not observe increases in larval deformities in any of our treatments.

Perhaps the most significant finding in these studies is that Cu, Ni, and Se resulted in similar bioaccumulation in *C. dilutus* and fathead minnow tissues when alone, in combination, or

when in a more complex MME mixture. These bioaccumulation patterns occurred even with some variation in Cu, Ni, and Se concentrations and differences in water chemistry across treatments. To our knowledge, this is the only study to have examined and compared the bioaccumulation patterns in fish between single metals, metal mixtures, and a complex MME under simultaneous waterborne and dietary exposures. These types of exposures are extremely complex and predicting responses to multiple mixtures is difficult, although necessary for environmental monitoring and risk assessment (Norwood et al. 2003). The metals we examined (Cu, Ni, and Se) do not appear to influence the bioaccumulation of each other in more complex mixtures (either metal mixture or MME), particularly in high hardness water conditions. However, MMEs contain a variety of other metals which may still contribute to additive or more than additive effects on fish.

Other metals, such as Cd and Zn have been shown to contribute to reduction in fathead minnow egg production when in combination with Cu in water at moderate hardness (~200 mg/L as CaCO3) (Eaton 1973). Effects on fathead minnow eggs for zinc alone have been reported at concentrations of approximately 145 µg/L in soft water (Benoit and Holcombe 1978). However, Cd and Zn were not elevated in 45% PWE, and therefore were not examined in the present studies. In moderately hard water (~200 mg/L as CaCO3), with Zn concentrations of ~20 µg/L and Cd at <1 µg/L, Cu concentrations of approximately 34 µg/L and above have been shown to reduce fathead minnow egg production (Mount 1968). These Cu, Zn, and Cd concentrations were nearly identical to the mixed metals component of the current study, further suggesting that hardness is a major factor responsible for reducing toxic responses in MMEs. Whether these effects occur due to other metals and bioaccumulation should be explored in future studies.

Despite the similar patterns of Cu, Ni, and Se bioaccumulation, reproductive impacts were not comparable between metal treatments and the 45% PWE.

2.4.6 Other possible causes of effects

As we found no evidence indicating that Cu, Ni, or Se, either alone or in mixture, contributed to reproductive impairment in fathead minnows during exposure to 45% PWE, it is possible that other metals or elements were responsible for effects. The concentration of B, Ba, Li, Rb, Ca, and Na tend to be greater in 45% PWE than RW in the present studies, which was also observed in previous studies (Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a). However, B, Ba, Li, and Rb are generally not believed to cause toxicity at low concentrations. Ca and Na were also present almost at similar concentrations in all treatments, including the single metal and metal mixture exposures that did not cause reproductive effects in fathead minnows, therefore likely were not responsible for negative effects in the 45% PWE.

Interestingly, densities of *C. dilutus* were reduced by approximately 27% (study 2) and 53% (study 3) in 45% PWE, relative to RW, in two of the three present studies. The reduction in fathead minnow egg production also occurred simultaneously with the reduction in *C. dilutus* densities in those 45% PWE exposures. Fish can be directly impacted through impaired physiology from the accumulation of toxic metals. However, they may also be affected indirectly through altered prey abundance or diversity - a consequence of metal contamination in the aquatic environments (Campbell et al. 2003, Rasmussen et al. 2008). Densities of *C. dilutus* have been reported to decrease during exposure to 45% PWE in several previous studies (Hruska and Dubé 2004, Hruska and Dubé 2005, Rozon-Ramilo et al. 2011a). Therefore, the role in food quantity should be examined in future studies to evaluate its role in metal bioaccumulation and influencing fathead minnow egg production.

2.4.7 Conclusion

Although we were not able to establish whether a specific metal is contributing to reproductive effects in fathead minnows, this research has reduced the likelihood that any of the three metals we examined plays a significant role. There is some evidence that suggests that, under certain circumstances, Cu, Ni, and Se can be responsible for reproductive effects in fish, however, under conditions similar to 45% PWE, single and mixed metals exposures alone did not result in any significant reproductive changes. Still, the 45% PWE caused decreased egg production in two of our three studies. In fact, concentrations of Cu and Se were low in the 45% PWE during the mining shutdown in one of our studies, however a decrease in fathead minnow egg production was still observed. This provides further evidence that Cu and Se are not responsible for reproductive effects in 45% PWE. Although water chemistry parameters and metal concentrations were marginally variable between some of the treatments, tissue bioaccumulation patterns were similar between our single metals, mixed metals, and 45% PWE fathead minnows, and generally low overall. Thus, it appears that exposure and bioaccumulation of these metals probably do not play a significant role in inducing reproductive effects in fish. As water chemistry parameters are known to influence toxicity of metals, artificially manipulating these parameters would be beneficial in order to further examine the role of metal bioaccumulation and toxicity in MME effluents.

3 CHAPTER 3[a]

THE INFLUENCE OF FOOD QUANTITY ON METAL BIOACCUMULATION AND REPRODUCTION IN FATHEAD MINNOWS (*PIMEPHALES PROMELAS*) DURING CHRONIC EXPOSURES TO A METAL MINE EFFLUENT

[a] This chapter examines the role of food quantity in influencing toxic responses of fish during chronic exposures to a Canadian metal mine effluent. The purpose of Chapter 3 was to investigate indirect factors of toxicity (i.e., food quantity – a potential causative factor of toxicity) and its role in causing metal bioaccumulation and reproductive impairment in fathead minnows during chronic exposure to the metal mine effluent. Food quantity was identified as a potentially important factor in contributing to toxic responses in fish observed in Chapter 2. This chapter has been published in the journal of Ecotoxicology and Environmental Safety, 2013, 91:188-197 under joint authorship with Som Niyogi (University of Saskatchewan) and Monique G. Dubé (Canadian Rivers Institute).

3.1 INTRODUCTION

Toxic responses in fish exposed to elevated concentrations of metals are influenced by many environmental (biotic and abiotic) factors. Numerous studies have examined the influence of abiotic factors, such as the physico-chemical parameters of water (e.g., hardness, pH/alkalinity, dissolved organic carbon, temperature), on metal bioavailability and toxicity to aquatic organisms including fish (see Niyogi and Wood 2004, Niyogi and Wood 2003, Wang 1987 for reviews). The current understanding of the role of water chemistry on metal bioavailability to aquatic organisms provide the mechanistic foundation of the biotic ligand model (BLM), which has emerged as a practical tool for risk assessment of metals in aquatic ecosystems (Di Toro et al. 2001, Niyogi and Wood 2004, Paquin et al. 2000, Santore et al. 2001). In addition to the abiotic factors, biotic factors (e.g., diet) can also influence metal bioavailability and toxicity to aquatic organisms (Niyogi and Wood, 2003). However, the current knowledge on the role of diet in influencing metal toxicity, particularly under environmentally relevant exposure scenarios (e.g., variable food availability, metal mixtures) is extremely limited. In this context, the effect of exposure to metal-impacted diet on reproductive performance in fish has been identified as a priority area of future research (Clearwater et al. 2002).

In general, dietary exposure of metals is known to be an important source of metal uptake and bioaccumulation in fish (Boyle et al. 2011, Bury et al. 2003, Croteau and Luoma 2009, Mathews and Fisher 2009). However, the link between dietary metal exposure and toxic responses in fish inhabiting metal-impacted environments is not very well understood, although a few studies have reported reduced growth and survival (Farag et al. 1999, Woodward et al. 1994, Woodward et al. 1995) and decreased reproductive output (Boyle et al. 2008) in fish when treated with metal-contaminated natural prey species. The characterization of toxic responses in

fish during dietary metal exposure is often confounded by reduced food consumption (loss of appetite or reduced food availability) in fish (Clearwater et al. 2002). Fish can be directly impacted (via impaired physiology and/or behaviour) due to the toxicity of accumulated metals, however they may also be affected indirectly (via food chain) due to altered prey abundance or diversity - a consequence of metal contamination in the aquatic environment (Campbell et al. 2003, Rasmussen et al. 2008). Indirect effects such as decreased prey abundance can also cause reduced physiological and/or reproductive fitness in the predator species.

In complex mixtures of metals, such as metal mine effluents (MMEs), understanding the causes of effects and separating the direct effects from indirect ones can be difficult. In field monitoring studies as well as mesocosm experiments, MMEs have been implicated for increased metal bioaccumulation in aquatic invertebrates and fish (Maret et al. 2003, Weber et al. 2008) and impaired reproductive capacity in fish (Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a, 2011b). In addition, decreased richness and abundance of benthic invertebrates and fish have been reported in areas downstream of MME discharge (Maret et al. 2003). The exposure to MMEs has also been found to cause reduced emergence, hatching success and survival of benthic invertebrate prey species (*Chironomids*) in laboratory exposures (Hruska and Dubé 2004, Hruska and Dubé 2005). These studies suggest that metal accumulation via water and diet as well as decreased prey abundance may both contribute to toxic responses in fish living in MME-impacted environments. Interestingly, dietary exposure appears to be a particularly important factor in impairing reproductive fitness of fish during exposures to MMEs. For example, reduction in egg production in MME-exposed populations of fathead minnows (*Pimephales promelas*) were reported to be significantly greater in the multi-trophic exposure (exposure via both water and diet) compared to that in the water-only exposure (Rickwood et al. 2006a). The

increased reproductive toxicity in fish during multi-trophic exposure was mediated either by poor food quality (metal-contamination), altered prey abundance, or the combination of both factors.

Using a multi-trophic mesocosm approach, we have shown previously that food quality can be an important factor in modulating reproductive capacity of fathead minnows during exposure to MMEs. This has been demonstrated through observed differences in egg production between fish that consumed metal-contaminated food and fish that consumed reference food, in either reference or MME exposure water (Rozon-Ramilo et al. 2011b). However, the role of food quantity in influencing egg production of fathead minnows has not yet been examined, despite the evidence that MMEs can decrease the density of *Chironomids* (the food source for fish in our multi-trophic mesocosms) (Hruska and Dubé 2004, Hruska and Dubé 2005, Rickwood et al. 2006a). Therefore, the objective of the present study was two-fold: (i) to examine whether the reproductive performance (egg production) of fathead minnows is affected by reduced food quantity during chronic exposures to an environmentally relevant MME; and (ii) to evaluate whether differences in food abundance influence the metal accumulation in target tissues of fathead minnows exposed to MME. A two factor experimental design was employed to determine whether the food quantity (normal *vs.* reduced ration of *Chironomids*) or the treatment water (reference water *vs.* MME) would play a more significant role in influencing responses in fish. It was hypothesized that fish consuming greater quantities of contaminated food during MME exposure would have greater tissue-specific metal burdens relative to that in fish consuming lesser quantities of contaminated food. It was also hypothesized that fish with greater tissue-specific metal burden would have greater decreases in egg production relative to that in fish with less tissue metal accumulation.

3.2 MATERIALS & METHODS

3.2.1 Experimental design and setup

The present study was performed at the University of Saskatchewan between February and April 2012. Experimental design and fish care were approved by the University of Saskatchewan Animal Care Committee which followed the Canadian Council on Animal Care protocols. Ten to twelve month old fathead minnows were obtained from in-house cultures for use in the fathead minnow reproductive bioassay. This reproductive bioassay consisted of a *Chironomus dilutus* culturing phase, a seven day pre-exposure period, and a twenty-one day exposure period. Cultures of *C. dilutus* larvae, a typical prey species for fish, were used as the food source for fathead minnows during the exposure period. The experimental exposure consisted of four different treatments: (i) reference water and normal food (RW-NF), (ii) reference water and low food (RW-LF), (iii) 45% process water effluent and normal food (PWE-NF), and (iv) 45% process water effluent and low food (PWE-LF). Food rations (*C. dilutus*) in our previous fathead minnow studies have usually been ~1 g/pair/day (roughly 14-20% fish body weight) (Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a, Rozon-Ramilo et al. 2011b). However, normal food rations for the present study were slightly higher (~1.2 g/pair/day; approximately 20-30% fish body weight) than during previous studies in order to ensure that all fathead minnows, including the larger males, would consume sufficient quantities of food to reach satiation. In contrast, food ration in the low food treatments was 0.4 g of *C. dilutus* larvae per fathead minnow pair per day (~6-10% fish body weight), which was based on the lowest densities of *C. dilutus* larvae observed in our previous multi-trophic mesocosm studies with 45% PWE [equivalent to densities of ~40 larvae/feeding section (Rickwood et al. 2006), and ~36 larvae/feeding section (Ouellet et al. 2013a)]. Fathead minnows in reference water treatments

were fed *C. dilutus* cultured in reference water, whereas fathead minnows in 45% PWE treatments were fed *C. dilutus* cultured in 45% PWE.

The reproductive bioassay was performed using modified multi-trophic mesocosm streams (see Hruska and Dubé 2004, Rickwood et al. 2006a for detailed descriptions). Multi-trophic mesocosms are an accepted component of Environment Canada's Environmental Effects Monitoring (EEM) program. Mesocosm systems used for the present study included four tables (one table/treatment), each with five replicate streams for fathead minnow breeding and three larval streams for hatching larvae. Each replicate stream consisted of a 10.3 L circular polyethylene tank, and contained a breeding tile, a 2.5 cm layer of pre-rinsed silica sand, and a mesh cover to prevent fathead minnows from escaping. The three larval streams were also similar except that they did not contain the silica sand, breeding tile or a mesh cover (see descriptions in section 2.5). Water exchanges for each treatment table were carried out by pumping the treatment water with a March pump (Model LC-3CP-MD, March Manufacturing, Glenview, IL, USA) from an 85 L reservoir (set at 1 turnover/day) through an 8-port manifold, continuously into the five replicate streams and three larval streams. Each 85 L reservoir was continuously renewed (at a rate of 1 turnover/day) with a Viking/Pulsatron pump (Series E 240 GPD LEF 75A-PHC3, Viking Pump of Canada, Edmonton, AB, Canada) connected to a 1200 L polyethylene holding tank. Treatment waters (RW or 45% PWE) were mixed manually every 2-3 days in the 1200 L holding tanks.

3.2.2 Treatment waters

The treatment waters for this study consisted of RW and 45% PWE. RW was made up of a mixture of 65% reverse osmosis (RO) water and 35% dechlorinated Saskatoon City water in order to match conditions (hardness, alkalinity and pH) to Vermillion River water, Ontario,

Canada. Vermillion River water has been used as the RW in our past field-based mesocosm studies (Rozon-Ramilo et al. 2011a) due to its proximity to the metal mine and the Junction Creek watershed in Ontario, Canada (PWE receiving environment). PWE was shipped weekly to the University of Saskatchewan and diluted to 45% with the RW in order to match environmentally relevant conditions of the receiving stream system (Rozon-Ramilo et al. 2011b). The same treatment waters (RW and 45% PWE) were used both for culturing *C. dilutus* and the fathead minnow exposures, whereas for fathead minnow pre-exposure, only RW was used.

3.2.3 Culturing of Chironomus dilutus

C. dilutus were cultured in either RW or 45% PWE in order to provide uncontaminated food for fathead minnows in RW treatments or contaminated food for fathead minnows in 45% PWE treatments. The culturing process took thirty six days, and was similar to the protocol described by Hruska and Dubé (2004), modified slightly to increase the acclimation period in order to improve culturing success. First-instar larvae (newly hatched) are generally considered to be the most sensitive to metals (Béchard et al. 2008), and therefore were not exposed immediately to the 45% PWE. Egg sacs were isolated from in-house *C. dilutus* cultures over four days and distributed into holding tanks filled with RW and 2 cm of silica sand. Egg sacs from *C. dilutus* were held in the RW-only treatment for the first six days. Subsequently, holding tanks were refilled with either RW or 5% PWE for 24h, followed by RW or 20% PWE for a further 24h, in order to acclimate *C. dilutus* gradually to the appropriate treatment. Following 48h of initial acclimation, *C. dilutus* were exposed either to RW or 45% PWE, and water exchanges were conducted at a rate of 50% every second day for the remainder of the culturing period. Larval *C. dilutus* were fed a blend of Tetramin^{TM} (~1 g/egg sac) every second day throughout the culturing period, beginning at day 6. Fourth instar *C. dilutus* were collected,

weighed, and distributed into vials to provide either 0.4 g or 1.2 g wet weight food quantities for both RW and 45% PWE fathead minnows. Vials were frozen and stored until the *C. dilutus* were fed to the fathead minnows in respective exposure treatments. Ten additional 1 g samples of *C. dilutus* were also collected from the RW and 45% PWE treatments (n= 5 each), frozen, and shipped to Testmark Laboratories Ltd. (Sudbury, ON, Canada) for tissue metal analyses.

3.2.4 Pre-exposure period

The purpose of the pre-exposure period was to select capable breeding pairs of fathead minnows for the experimental exposures. The pre-exposure period for this study lasted seven days. Fathead minnow pairs (one male and one female) were randomly distributed to 10.3-L RW filled, polycarbonate streams (n=49) with a spawning tile, and were monitored daily for egg production. This consisted of checking each spawning tile for eggs, scraping any eggs present onto a petri dish, and photographing the eggs (Canon Powershot A620 digital camera mounted to a Vista Vision™ Model 48402-00 microscope, VWR International, Missisauga ON, Canada). Egg production in each tank was determined by counting the eggs in the photographs. During the pre-exposure period, fathead minnow breeding pairs were fed frozen fish food (Sally's bloodworms™, San Francisco Bay Brand, Inc., Newark, CA, USA) twice daily at a rate of approximately 10-14% body weight (~0.8g/pair). Water temperature was maintained at 25±2 °C and photoperiod was 16h:8h (light:dark). Water temperature, dissolved oxygen, conductivity and ammonia were monitored daily. At the end of the pre-exposure period, mean total egg production (# eggs/pair/day) was calculated for each pair of fathead minnow. The 20 fathead minnow breeding pairs with highest mean total egg production were selected for the exposure period and were distributed evenly among the four experimental treatments (mean of ~19-23 eggs/pair/day for each treatment, n=5) (see Appendix Figure A-1). This ensured compatibility in

fathead minnow breeding pairs across the treatments at the onset of the exposure period, and thus minimized the potential sources of variation in egg production.

3.2.5 Exposure period

The exposure period for this study lasted twenty-one days. Total lengths and body weights of fathead minnows were measured before introducing them into the streams. Fathead minnow breeding pairs were distributed into RW or 45% PWE treatments, and assigned into normal or low food ration treatments (n=5 for all four treatments). Frozen *C. dilutus* were thawed for 10-15 minutes, and subsequently fed to the experimental fish once daily.

Fathead minnow egg production was monitored daily as described previously. Ten eggs per brood were selected and analyzed with Image Pro Plus 6.1 (Media Cybernetics Inc., Maryland, USA) for egg size determinations. Eggs were also collected daily to evaluate fertilization rate, hatch rate, and larval deformities. Infertile eggs were either opaque, had a visibly precipitated yolk, or contained no yolk (Ankley et al. 2001). Following the photographing, eggs were transferred into the labelled egg cups (one cup for each brood), and placed in the larval streams of the respective treatments (RW or 45% PWE). Egg cups were made from PVC pipe with a screen mesh on the bottom which allowed water renewal within the cup. A single air-stone was also placed in each egg cup to agitate the eggs and prevent fungal growth, which is typical of fathead minnow eggs that have been removed from parental care (Divino and Tonn 2008). Non-viable or fungus infected eggs were removed from the egg cups daily. Once hatched, larvae were again placed on the petri dishes and photographed under the microscope to assess larval lengths and deformities. Deformities were assessed on all hatched larvae, as opposed to lengths which were measured on five hatched larvae, chosen randomly from each brood, with Image Pro Plus 6.1.

On the final day of the exposure period, fathead minnows were anesthetized with methane tricainesulfonate (MS-222), measured for total length and body weight, then dissected to obtain liver, gill, gonad, and the carcass tissues. All tissues were weighed, however, only female tissues were placed in pre-labelled sample bottles, frozen, and sent to Testmark Laboratories Ltd. (Sudbury, ON, Canada) for metal analyses. Female tissues were analyzed because they have been shown to accumulate greater concentrations of metals than males in field studies with PWE (Weber et al. 2008). Moreover, metal bioaccumulation in females is likely to be linked with effects on egg production and larval development.

3.2.6 Water quality and tissue analyses

During the exposure period, water quality measurements were taken daily from one stream chosen at random per treatment. Daily water quality measurements included temperature, dissolved oxygen (DO) and conductivity [YSI meter (Yellow Springs Instruments, Yellow Springs, OH, USA)], as well as ammonia (kit procured from Rolf C. Hagen, Edmonton, AB, Canada), pH (pH Meter, Oakton Instruments, Vernon, IL, USA), and alkalinity (kit procured from LaMotte Company, Chestertown, MD, USA). Weekly water samples were also taken on days 7, 14, and 21 to determine concentrations of anions, total metals (46 different elements), dissolved organic carbon (DOC), and total organic carbon (TOC) from each treatment. The weekly water samples were collected from one stream per treatment at random, in pre-labeled high density polyethylene (HDPE) sample bottles, placed in a cooler with ice, and shipped to Testmark Laboratories Ltd. (Sudbury, ON, Canada) where these analyses were carried out.

The weekly water samples were analyzed for total metals using inductively coupled plasma-mass spectrometry (ICP-MS), for Ca^{2+} and Mg^{2+} concentrations using ICP-MS without acidification [and converted to total water hardness expressed as the equivalent of calcium

carbonate ($CaCO_3$) in mg/L], for anions by Ion Chromatography, and for dissolved organic carbon (DOC) using a Dohrman TOC Analyzer. All these measurements were conducted in accordance with the analytical methodology of the American Public Health Association (APHA) and US Environmental Protection Agency (EPA).

ICP-MS with Microwave Digestion was performed to determine metal concentrations in *C. dilutus* and fish tissues. The quality control and quality assurance for the metal analyses were maintained using method blanks, positive controls, blank spikes and laboratory duplicates with water samples, as well as a certified reference material [DOLT-3 dogfish (*Squalus acanthias*) liver obtained from the National Research Council of Canada]. Percentage recovery for the elements analyzed varied between 90 to 110%.

3.2.7 Data analysis and statistics

Data were analyzed and plotted using IBM SPSS Statistics 20.0.0 (SPSS, Chicago, IL) and Sigmaplot® Version 11.0 (San Jose, CA, USA). Water chemistry and metal burdens in *C. dilutus* tissues were analyzed using one-way analysis of variance (ANOVA). Mean adult survival, changes in body weight from day 0 to day 21, condition factor [k = body weight (g) / total length^3 (cm^3) x 100], liver somatic index [LSI (%) = liver weight (g) / body weight (g) x 100], gonadal somatic index [GSI (%) = gonad weight (g) / body weight (g) x 100], mean total egg production (calculated as mean total # eggs/pair), mean egg size, mean larval length, mean larval deformities (%), mean fertilization success (%), and mean fathead minnow tissue metal concentrations per replicate were analyzed using two-way ANOVA, with food quantity and treatment water as the two independent factors. Cumulative total egg production (calculated as: # eggs per treatment each day, summed cumulatively for 21 days) and cumulative total spawning events were compared between each treatment by performing multiple, two-sample

Kolmogorov-Smirnov (KS) tests. The Shapiro-Wilk test was used to test parametric assumptions for normality and Levine's test was used to test for homogeneity of variance prior to one-way and two-way ANOVAs. Data that failed these assumptions were either transformed [arcsin(%) or $\log_{10}$] or analyzed using the non-parametric Kruskal-Wallis test. If significant differences were detected using one-way ANOVAs, Tukey's post-hoc test was then used to determine if differences were present between RW and 45% PWE treatments. If significant differences were detected using two-way ANOVAs, Tukey's post-hoc test was then used to determine if the differences were due to treatment water effects (i.e., RW *vs*. 45% PWE), food quantity effects (i.e., NF *vs*. LF), and/or their interaction effects. A significance level of α=0.05 was used for all statistical analyses.

3.3 RESULTS

3.3.1 General water quality

There were statistically significant differences in water hardness, pH, conductivity, ammonia, DOC, calcium, magnesium, sodium, sulfate and chloride between the 45% PWE and the RW treatments (Table 3-1). Other parameters, such as temperature and nitrate were not statistically different among any of the treatments.

3.3.2 Concentrations of metals in the exposure water and fish diet

A total of 41 metals were analyzed in experimental water and tissue samples. Among them, 11 metals (barium, boron, cobalt, copper, lithium, manganese, nickel, rubidium, selenium, strontium, thallium) were found to be significantly elevated in both 45% PWE treatment waters relative to the reference water (RW) treatments (Table 3-2). Interestingly however, not all metals that were elevated in 45% PWE treatment waters accumulated in the tissues of *C. dilutus* larvae. *C. dilutus* larvae that were cultured in 45% PWE water had significantly elevated

Table 3-1 – General water quality measurements sampled randomly from reference water normal food (RW-NF), reference water low food (RW-LF), 45% process water effluent normal food (PWE-NF), and 45% process water effluent low food (PWE-LF) treatments throughout the 21-day exposure period.

General Water Quality		RW-NF	RW-LF	PWE-NF	PWE-LF
Temperature	°C	24.2±0.1[a]	24.4±0.1[a]	24.3±0.0[a]	24.5±0.2[a]
pH	pH	7.9±0.0[a]	8.0±0.0[a]	7.4±0.0[b]	7.3±0.0[b]
DOC*	mg/L	1.27±0.03[ab]	1.13±0.09[b]	1.60±0.10[ac]	1.70±0.10[c]
Ammonia	mg/L	0.24±0.02[a]	0.15±0.02[a]	1.45±0.19[b]	1.76±0.14[b]
Chloride	mg/L	3.45±0.53[a]	3.64±0.57[a]	42.93±6.73[b]	40.93±4.81[b]
Conductivity	mg/L	156.3±7.3[a]	151.3±5.7[a]	1213.6±55.8[b]	1302.8±24.5[b]
Dissolved Oxygen	%	89.8±0.6[a]	89.4±0.6[a]	90.3±0.7[a]	89.7±0.7[a]
Nitrate	mg/L	0.29±0.03[a]	0.25±0.00[a]	0.84±0.24[a]	0.59±0.09[a]
Sodium	mg/L	9.19±0.88[a]	9.19±0.88[a]	54.87±3.02[b]	57.27±6.77[b]
Sulfate	mg/L	28.57±3.54[a]	28.33±4.41[a]	685.67±82.95[b]	695.33±58.33[b]
Total Hardness (as $CaCO_3$)	mg/L	47.87±2.96[a]	47.70±2.83[a]	538.67±18.26[b]	543.67±53.82[b]
Calcium	mg/L	10.55±0.83[a]	10.45±0.81[a]	189.33±7.54[b]	191.33±19.43[b]
Magnesium	mg/L	5.24±0.31[a]	5.27±2.45[a]	15.93±0.20[b]	15.97±1.33[b]

Values are mean ± standard error.

Sample sizes were n=21 for parameters that were measured daily (temperature, pH, ammonia, conductivity, and dissolved oxygen) and n=3 for parameters that were measured weekly (DOC, chloride, nitrate, sodium, sulfate, total hardness, calcium, and magnesium).

Means that do not share lowercase letters are statistically different from one another for that general water quality parameter (one-way ANOVA; Tukey HSD post hoc test, p<0.05)

*dissolved organic carbon

Table 3-2 – Concentrations of selected total metals measured from weekly samples of reference water normal food (RW-NF), reference water low food (RW-LF), 45% process water effluent normal food (PWE-NF), and 45% process water effluent low food (PWE-LF) treatments during the 21 day exposure period.

Element		RW-NF	RW-LF	PWE-NF	PWE-LF
Barium	µg/L	9.87±0.30[a]	9.83±0.38[a]	15.37±0.69[b]	16.47±1.65[b]
Boron	µg/L	14.07±3.07[a]	14.67±2.73[a]	36.83±3.71[b]	37.00±4.13[b]
Cadmium	µg/L	≤0.10±0.00[a]	≤0.10±0.00[a]	≤0.10±0.00[a]	≤0.10±0.00[a]
Cobalt	µg/L	≤0.50±0.00[a]	≤0.50±0.00[a]	1.24±0.10[b]	1.17±0.10 [b]
Copper	µg/L	16.43±0.55[a]	13.83±0.82[a]	69.93±4.03[b]	76.53±11.96[b]
Lithium	µg/L	3.40±0.90[a]	3.53±1.03[a]	22.67±0.33[b]	24.00±0.58[b]
Manganese	µg/L	0.50±0.00[a]	0.50±0.00[a]	4.30±0.40[b]	4.70±0.59[b]
Nickel	µg/L	4.30±0.17[a]	1.53±0.09[a]	34.40±2.95[b]	33.53±2.47[b]
Rubidium	µg/L	≤0.50±0.00[a]	0.50±0.00[a]	19.17±0.72[b]	20.73±1.79[b]
Selenium	µg/L	≤0.50±0.00[a]	≤0.50±0.00[a]	6.50±0.23[b]	6.17±0.62[b]
Strontium	µg/L	59.87±6.75[a]	58.67±6.43[a]	303.00±24.66[b]	320.33±41.91[b]
Thallium	µg/L	≤0.05±0.00[a]	≤0.05±0.00[a]	0.13±0.01[b]	0.11±0.03[b]
Zinc	µg/L	16.77±5.62 [a]	6.83±1.22[a]	29.27±17.33[a]	9.33±1.56[a]

Values are mean ± standard error.

Sample sizes were n=3.

Means that do not share lowercase letters are statistically different from one another for that element (one-way ANOVA; Tukey HSD post hoc test, p<0.05)

concentrations for only 5 metals (copper, nickel, rubidium, selenium, and thallium) relative to the larvae cultured in RW (n=5 for each treatment) (Figure 3-1), with selenium exhibiting the highest magnitude of increase (~100 fold) followed by thallium (~13 fold), nickel (~10 fold), copper (~ 6 fold), and rubidium (~4 fold).

3.3.3 Fathead minnow survival and morphometrics

There were no statistically significant differences in survival rates of fathead minnows among any of the treatments (two-way ANOVA; p>0.05). A single female died on day 21 in the PWE-LF treatment. There was a statistically significant food quantity effect on the changes in body weights from day 0 to day 21 in both male and female fathead minnows, as fish in low food ration treatments weighed significantly less on day 21 than day 0, irrespective of 45% PWE exposure (Figure 3-2A). The alterations in fish morphometrics on day 21 are presented in Figure 3-2B. There was a treatment water effect on male gonadosomatic indices (GSI), with significantly higher values in both 45% PWE treatments relative to that in either RW treatment. In contrast, a food quantity effect was recorded for female GSI, male and female liver somatic indices (LSI), and male condition factor (K), as each of these morphometric parameters were significantly greater in normal food ration treatments relative to that in low food ration treatments, irrespective of 45% PWE exposure. There was no difference in female K values among any experimental treatments.

3.3.4 Tissue specific metal accumulation in female fathead minnows

3.3.4.1 Gonads

Metal accumulation profiles in the gonads of female fathead minnows across the different experimental treatments are presented in Figure 3-3A. There were significant treatment water effects for rubidium and selenium, and the concentrations of both metals were elevated in fish

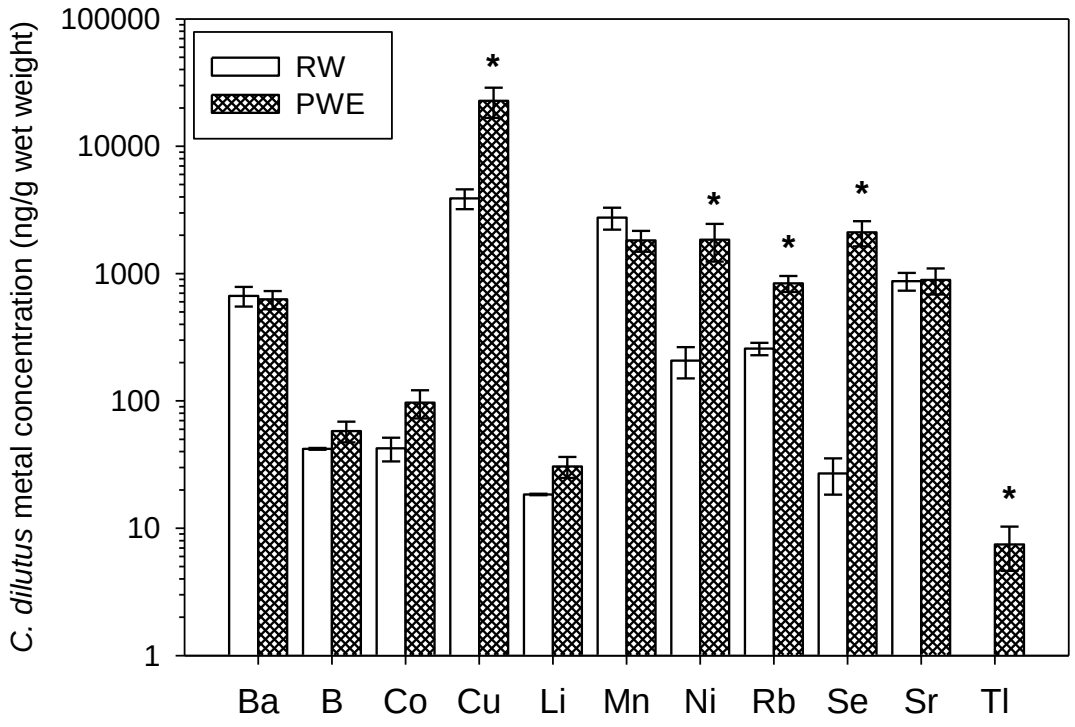

Figure 3-1 – Concentrations of different metals in *C. dilutus* larvae that were cultured either in reference water (RW) or 45% process water effluent (PWE), and used as the food source [0.4 g (low food ration) *vs*. 1.2 g (normal food ration)] during the exposure period. Values are means ± S.E. (n=5). Asterisks represent significant differences between the two treatments for any particular metal (one-way ANOVA; $p<0.05$).

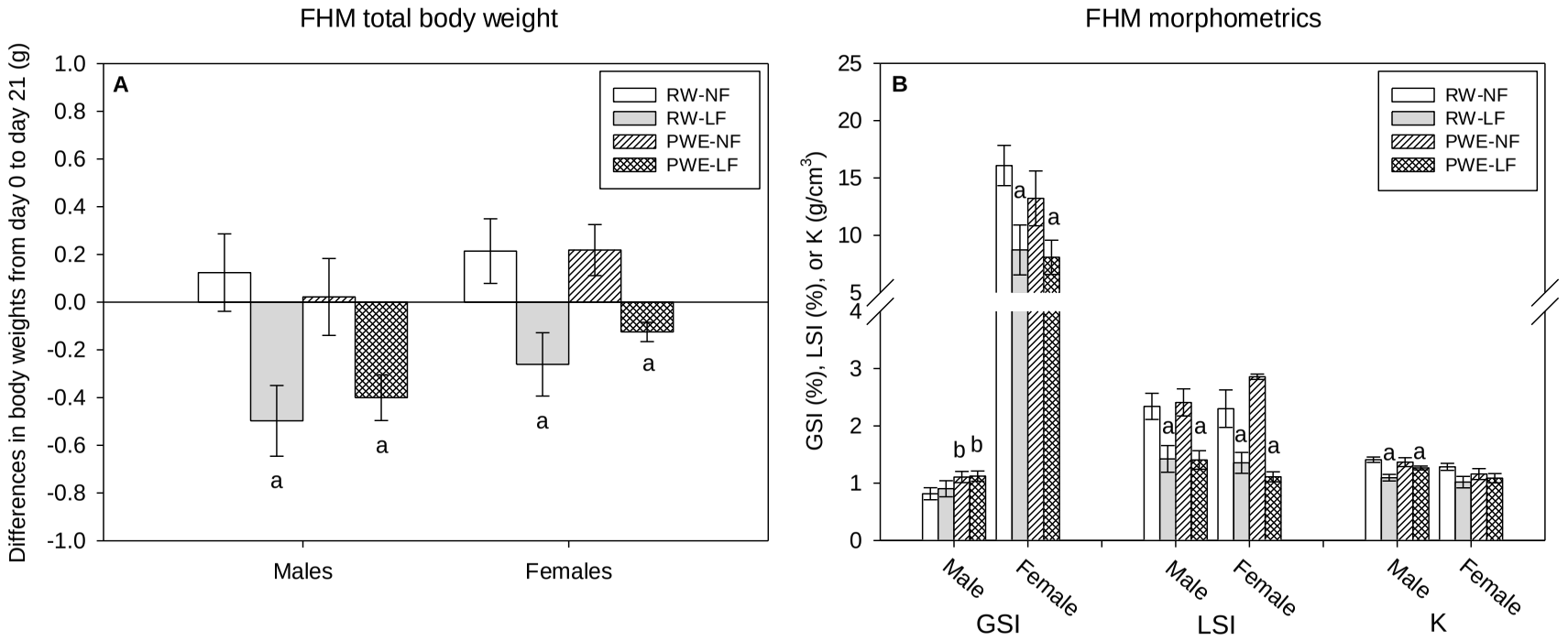

Figure 3-2 – Differences in body weights for male and female fathead minnows (FHMs) between the end (day 21) and beginning (day 0) of exposure in different experimental treatments (A), and gonadosomatic indices (GSI), liver somatic indices (LSI), and condition factor (K) in male and female FHMs in different experimental treatments at the end of 21 day exposures (B). Values are means ± S.E. Lowercase "a" indicates a food quantity effect and lowercase "b" indicates a treatment water effect, for any particular morphometric index (two-way ANOVA; $p<0.05$). Sample size (n) was 5 for both sexes in all treatments, except for females in PWE-LF treatment where the sample size was 4.

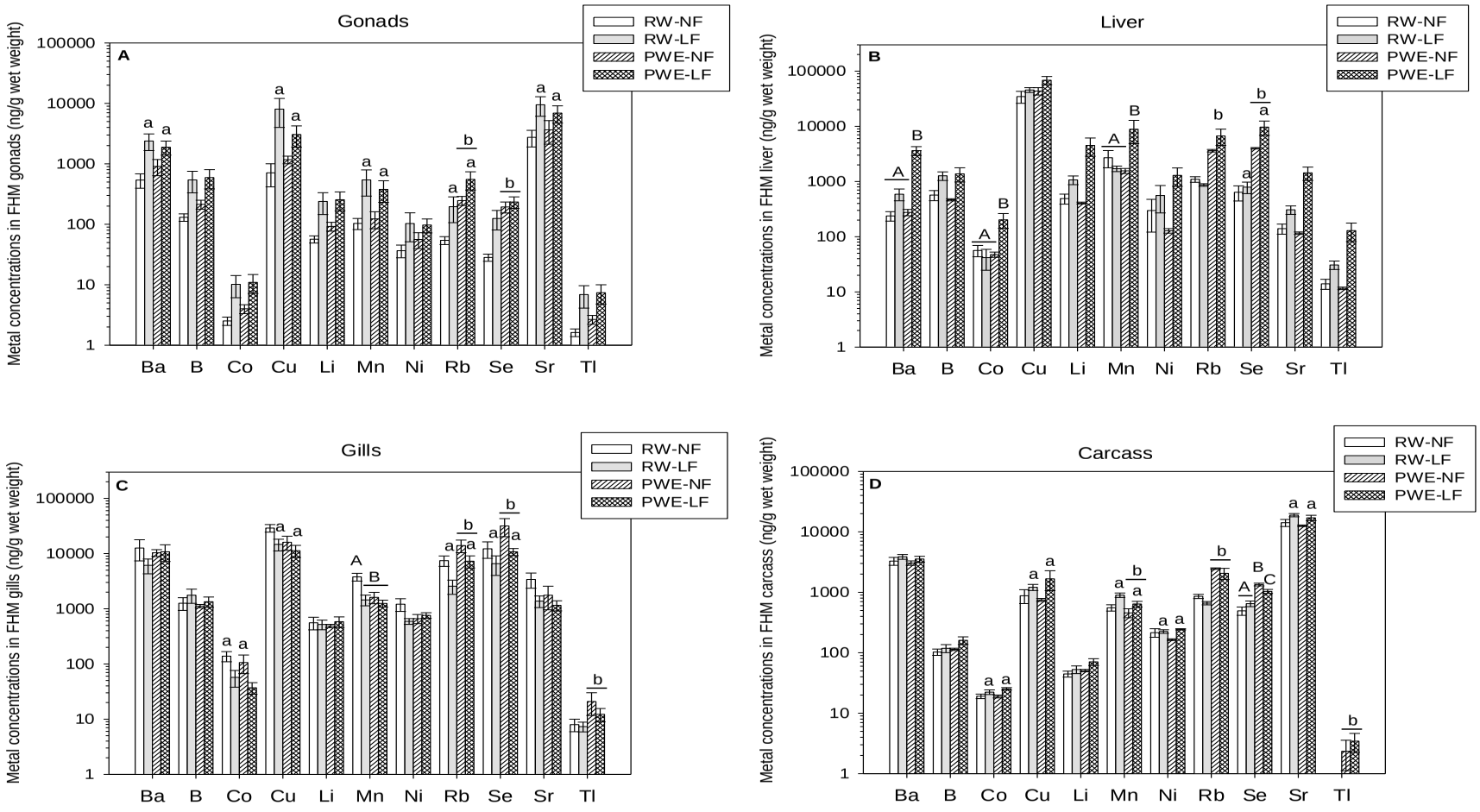

Figure 3-3 – Concentrations of various metals in the gonads (A), liver (B), gills (C), and carcass (D) of female fathead minnows (*P. promelas*) in different experimental treatments at the end of exposure (day 21) . Values are means ± S.E. Lowercase "a" indicates a food quantity effect and lowercase "b" indicates a treatment water effect, for any particular metal (two-way ANOVA; $p<0.05$). Uppercase letters indicate an interaction effect between food quantity and treatment water, where statistically significant differences among treatments are indicated by different letters (Tukey HSD post-hoc test; $p<0.05$). Sample size (n) was 5 in all treatments, except in PWE-LF treatment where the sample size was 4.

exposed to 45% PWE relative to RW, independent of the food quantity. There were also significant food quantity effects for barium, copper, manganese, rubidium, and strontium in gonads, each metal with a higher concentration in fish from the LF treatments relative to NF treatments, independent of treatment water type. No significant interaction between treatment water and food quantity was recorded for any metals which accumulated in female fathead minnow gonads. There were no statistically significant differences (two-way ANOVA; $p>0.05$) in concentrations of boron, cobalt, lithium, thallium or nickel in gonad tissues among the four different treatments.

3.3.4.2 Liver

Metal accumulation profiles in the livers of female fathead minnows across the different experimental treatments are presented in Figure 3-3B. There were significant treatment water effects for rubidium and selenium, as the concentrations of both metals were greater in female fathead minnows exposed to 45% PWE relative to RW, independent of food quantity. In addition, a significant food quantity effect was observed for selenium, which was present in higher concentration in the livers of female fathead minnows from the low food ration treatments relative to the normal food ration treatments, independent of the treatment water type. There were significant interaction effects between treatment water type and food quantity for barium, cobalt, and manganese in fish liver tissues, and the hepatic concentration of each of these metals was elevated in the PWE-LF treatment relative to that in all other treatments. No statistically significant differences (two-way ANOVA; $p>0.05$) in concentrations of lithium, nickel, strontium, thallium, boron, or copper were observed in the fish liver tissues among the different treatments.

3.3.4.3 Gills

Metal accumulation profiles in the gills of female fathead minnows across the different experimental treatments are presented in Figure 3-3C. There were significant treatment water effects for rubidium, selenium, and thallium in gills tissues. The concentration of each of these metals was significantly higher in the 45% PWE treatments relative to the RW treatments, independent of food quantity. In addition, a significant food quantity effect was observed for cobalt, copper, rubidium, and selenium in gill tissues. Each of these metals was observed at elevated concentrations in female fathead minnow gills from the normal food ration treatments relative to the low food ration treatments, independent of the treatment water type. There was a significant interaction effect between treatment water and food quantity for manganese, with higher gill concentration in fish from the RW-NF treatment relative to all other treatments. There were no statistically significant differences (two-way ANOVA; $p>0.05$) in concentrations of boron, lithium, barium, nickel, or strontium in the gill tissues among any treatments.

3.3.4.4 Carcass

Metal accumulation profiles in the carcass of female fathead minnows across the different experimental treatments are presented in Figure 3-3D. There was significant treatment water effects for rubidium, and thallium in the female fathead minnow carcasses, and the concentrations of both metals were elevated in the 45% PWE treatments relative to the RW treatments, independent of food quantity. A significant food quantity effect was recorded for cobalt, copper, manganese, nickel, and strontium, and their concentrations were greater in the low food ration treatments relative to the normal food ration treatments, independent of treatment water type. There was also a significant interaction effect between treatment water type and food quantity for selenium, which was found at elevated concentration in female fathead minnow

carcasses in the PWE-NF treatment relative to the PWE-LF treatment, with no significant difference between the two RW treatments. No statistically significant differences (two-way ANOVA; $p>0.05$) were recorded in the concentrations of boron, lithium and barium in female fathead minnow carcasses among any treatments.

3.3.5 Fecundity

Mean total egg production per fathead minnow breeding pair in all of the treatments is presented in Figure 3-4. There was no treatment effect observed for fathead minnow mean total egg production (as # eggs/pair/treatment) during the exposure period, however a statistically significant effect of food quantity was recorded (two-way ANOVA; $p=0.05$). Mean egg production over 21 days of exposure decreased in the low food ration treatments irrespective of 45% PWE exposure. The mean egg production decreased by 59% from the normal food ration to low food ration treatments in reference water, whereas the magnitude of decrease from the normal to low food quantity treatments was 88% in 45% PWE water. There was no interaction effect (treatment water x food) for mean total egg production (two-way ANOVA; $p=0.413$) among any treatments.

There were statistically significant differences in the distributions of cumulative total egg production among all treatments (two sample Kolmogorov-Smirnov; $p\leq0.002$ for all pairwise comparisons) (Figure 3-5). Egg production in the normal food ration treatments was greater relative to that in the low food ration treatments, following a pattern of PWE-NF > REF-NF > RW-LF > PWE-LF. There were, however, no statistically significant differences in the distributions of cumulative total spawning events in fathead minnow among any treatments (two sample Kolmogorov-Smirnov; $p>0.05$ for all pairwise comparisons) (data not shown). There was also no statistically significant difference in (two-way ANOVA; $p>0.05$ for each) treatment

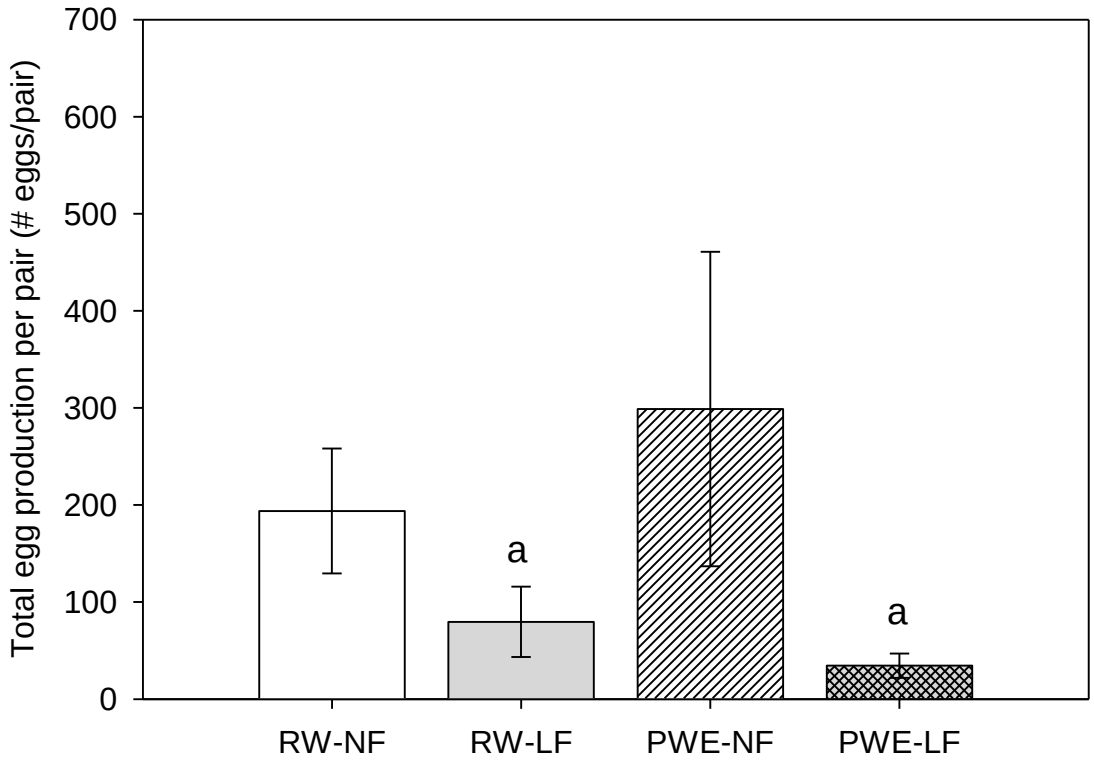

Figure 3-4 – Total egg production per fathead minnow (*P. promelas*) breeding pair (# eggs/pair) in reference water normal food (RW-NF), reference water low food (RW-LF), 45% process water effluent normal food (PWE-NF), and 45% process water effluent low food (PWE-LF) treatments over the exposure period of 21 days. Values are means ± S.E. (n=5). Lowercase "a" indicates a food quantity effect (two-way ANOVA; $p<0.05$).

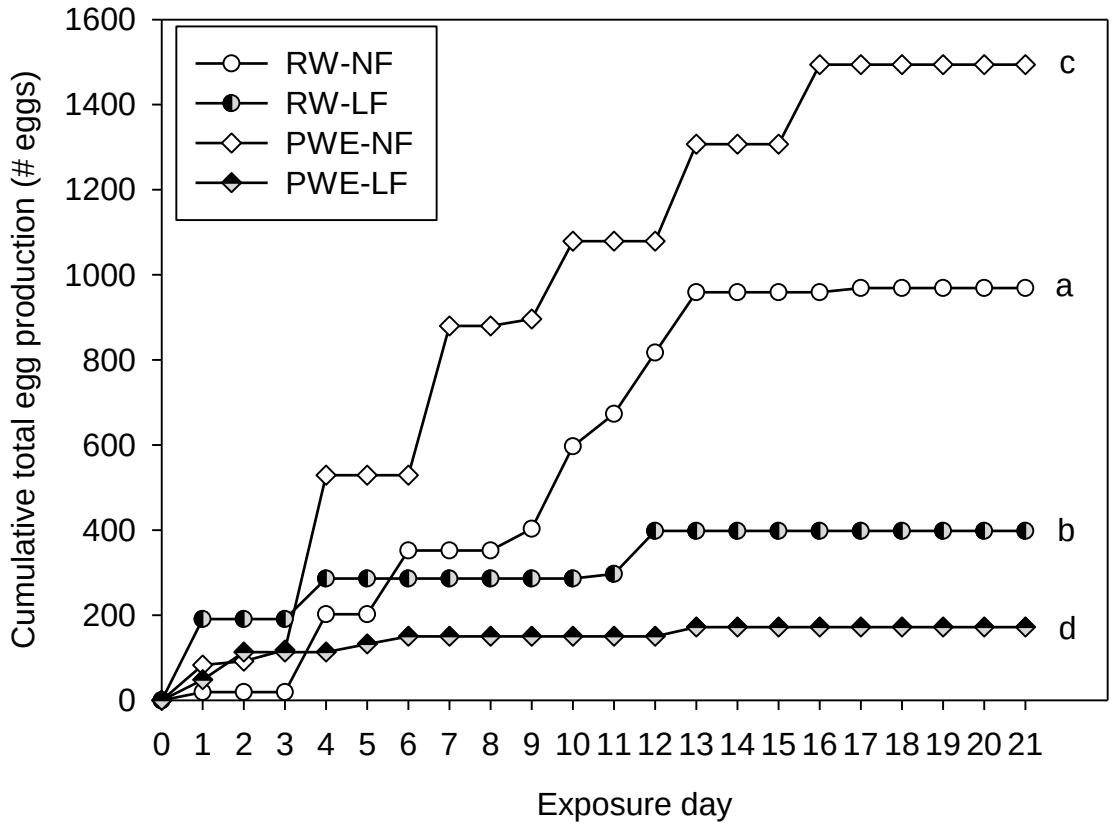

Figure 3-5 – Cumulative total egg production (# eggs) of fathead minnow (*P. promelas*) breeding pairs in reference water normal food (RW-NF), reference water low food (RW-LF), 45% process water effluent normal food (PWE-NF), and 45% process water effluent low food (PWE-LF) treatments over the exposure period of 21 days. Distributions that do not share letters are statistically different from one another (two-sample Kolmogorov-Smirnov; $p<0.05$ for all pairwise comparisons).

water, food quantity or their interaction effect on egg sizes, hatch rate, fertilization success, larval lengths, or larval deformities in fish (data not shown - see Appendix Figure A-2 for rate of larval deformities, Appendix Figure A-3 for hatch rate, and Appendix Figure A-4 for egg sizes of fish).

3.4 DISCUSSION

The results from this study demonstrate the importance of food quantity in influencing metal accumulation patterns and reproductive performance (egg production) in fish during exposures to complex metal mixtures. Metal mine effluents from base metal mining operations generally contain elevated levels of many different elements including metals (e.g., copper, nickel, selenium) that are toxic to fish at fairly low concentrations (Rozon-Ramilo et al. 2011a). In the present study, 45% PWE contained elevated levels of 11 metals that fathead minnows were exposed to through the water. Five of these metals also accumulated in the *C. dilutus*, which was the food source in our study, suggesting their potential bioavailability to fish through the diet as well. Since the present study included consistent levels of waterborne metal exposure to fish in both 45% PWE treatments (PWE-NF and PWE-LF), it was reasonable to assume that differential tissue-specific metal accumulation and egg production patterns would occur due to quantitative differences in the availability of metal-contaminated diet.

Our study design was expected to elicit a significant interaction between food quantity and complex metal-mixture on tissue metal accumulations, since fish exposed to reference and 45% PWE water were fed variable quantities of *C. dilutus* larvae cultured in the reference and 45% PWE water, respectively. One of our major hypotheses in the present study was that the treatment of fish with a relatively higher food ration of metal-contaminated food would lead to increased tissue-specific accumulation of metals, as previously suggested by Mathews and Fisher

(2009). Interestingly, our findings did not support our hypothesis, except with selenium concentrations in the carcass of fish which increased in the PWE-NF treatment relative to the PWE-LF treatment. Selenium exposure in fish occurs primarily through the diet (Janz et al. 2010) and accumulation occurs in almost all major tissues of fish (Misra et al. 2012). In the present study, selenium concentrations increased in all tissues except the gills in PWE-exposed fish, irrespective of food quantity. Although the selenium level was significantly elevated both in the water and diet in the PWE exposures relative to the RW exposures, the increase of selenium concentration in the liver and carcass, as opposed to the gills, further suggests that diet was the primary source of elevated selenium tissue burden in PWE exposures. The accumulation of selenium in liver and carcass of fish could be a potential cause for toxicity, as selenium is known to transfer from these tissues to eggs and cause reproductive impairment (Janz et al. 2010). Although we observed a marginal increase of gonad (ovary) selenium level in the PWE-NF and PWE-LF treatments, egg production was decreased only in the latter, thereby suggesting that the effect was probably not mediated by selenium. Moreover, the selenium level in the ovary of PWE-exposed fish [<1.44 µg/g dry weight, based on ~75% moisture content (Couture and Rajotte, 2003)] was well below the currently proposed threshold level of ovarian selenium (>10 µg/g dry weight). In addition to selenium, increased accumulation of rubidium in the liver and carcass, as well as increased accumulation of thallium in the gills and carcass, were recorded in PWE-exposed fish relative to fish in RW exposures, irrespective of food quantity. The implications of rubidium and thallium accumulation in target tissues are difficult to assess since the toxic mechanisms of these metals in fish are not known. Nevertheless, reproductive effects observed in PWE-exposed fish is probably not linked with the increased tissue burden of either

rubidium or thallium since egg production decreased only when fish were fed with a lower food ration.

On the other hand, an interaction between food quantity and complex metal-mixture was recorded for barium, cobalt and manganese accumulation, although this effect was only evident in the liver. Notably, however, the concentrations of these metals in the liver of fish increased significantly in the PWE-LF, but not in the PWE-NF, treatment. This was again contrary to our assumption that a higher ration of metal-contaminated food would lead a greater tissue metal burden in fish. Since barium, cobalt, and manganese were each not elevated in the *C. dilutus* larvae, it is likely that the accumulation of these metals occurred in fish via water. The accumulation of these metals across the gills occurred possibly due to mimicry of essential ions, as fish might have upregulated the waterborne uptake of ions in order to compensate for low dietary intake. The accumulation of barium in the livers has been documented in fish from metal contaminated sites (Jabeen et al. 2011), but its toxicity in fish is largely unknown. Cobalt and manganese are essential metals and are not expected to cause toxicity in fish unless their concentration in the body is extremely high. Overall, our results suggest that the accumulation of barium, cobalt and manganese in the liver of fish in the PWE-LF treatment was modest (3-4 fold increases compared that in the reference water fish), and played a minor role in inducing reproductive impairment in fish.

Overall, the tissue-specific metal accumulation profile in fathead minnow observed in the current study is consistent with the previous mesocosm studies with 45% PWE (Rickwood et al., 2006; Rozon-Ramilo et al., 2011a, 2011b), despite the fact that the concentrations of different metals are often quite variable in the metal mine effluents. The general lack of interaction between food quantity and metal-mining effluent can be attributed largely to differences in the

condition of fish among the experimental treatments. The differences in body weights and fish morphometrics between normal food and low food ration treatments observed in our experiment are consistent with general relationships between nutritional status, energy allocation and typical condition indices (Alonso-Fernández and Saborido-Rey 2012, Wuenschel et al. 2012). While comparing the effect of variable food quantity on fish health, the availability of higher food ration is expected to generate increased energy and lipid deposits, ultimately resulting in greater growth, GSI, LSI and K, relative to the low food ration. In our previous studies with 45% PWE, body weights and K of fathead minnows have been found to be influenced by food quality (contaminated *vs*. uncontaminated food) (Rozon-Ramilo et al. 2011b), although unlike in the present study, fathead minnow morphometric parameters have usually been much less affected during multi-trophic exposures (Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a). This is because the present study examined the effects in fish under differences in food ration (1.2 g/pair *vs*. 0.4 g/pair) that was somewhat higher than the previous studies. The decrease in *C. dilutus* densities among treatments in multi-trophic studies has generally been in the range of ~30-50% [1.0 g/pair (reference) *vs*. 0.5-0.7 g/pair (PWE) on day 21 of the exposure] (Rickwood et al. 2006; Rozon-Ramilo et al. 2011a).

In our study, diet quantity and its influence on fish morphometrics appears to be the primary factor responsible for influencing metal accumulation patterns in fish, particularly in the gonads and carcass, regardless of the treatment water. The growth of fathead minnows in the normal food ration treatments and the concurrent reduction in body weight as well as GSI and K values in the low food treatments explains much of the differences in metal accumulation in the gonads and carcass recorded in our study. These body and tissue weight changes in fathead minnow suggest a growth dilution effect in the normal food ration treatments, and an antithetical

effect in the low food ration treatments where the concentration of metals increased due to the reduction in tissue mass. Somatic growth dilution occurs when biomass increases at a faster rate than metal bioaccumulation (Karimi et al. 2010) and is seldom factored into the evaluation of metal accumulation in fish. Tissue copper concentration, in particular, has been found to be influenced by growth dilution in rainbow trout during dietary exposure (Kamunde et al. 2002, Kamunde and Wood 2003). Thus, it is reasonable to suggest that the somatic growth dilution was primarily responsible for lower concentrations of some metals in the gonads and carcass (e.g., barium, copper, manganese and strontium in the gonads; cobalt, copper, manganese, nickel and strontium in the carcass) in the normal food ration treatments, independent of the exposure water. In contrast, metal concentrations in the liver appear to be relatively much less affected by the somatic growth dilution, and more influenced by the decreased efficiency to eliminate metals due to lower food consumption during exposure to the PWE. This is indicated by the elevated metal concentrations in the fathead minnow liver in PWE-LF treatments despite lower and similar LSI value relative to the PWE-NF and RW-LF treatments, respectively. Fish allocate significant energy resources to metal detoxification and elimination when living in the metal-contaminated environments (Hashemi et al. 2008, Öner et al. 2009, Smith et al. 2001). It appears that unlike in PWE-NF treatment, fish in the PWE-LF treatment were probably less efficient in eliminating these metals from the liver (the primary site of metabolism for metals) due to energetic deficiencies, which is apparent from their lower LSI value (an index of reduced energy storage).

In the present study, we also hypothesized that fish with higher tissue metal burden would elicit greater reduction in cumulative total egg production during exposure to complex metal-mining effluent. Although we recorded increased tissue burden of some metals in PWE-exposed

fish, particularly when treated with a low food ration (as described above), the decrease in total egg production appears to be primarily induced by reduced food ration rather than increased tissue metal burden. Fish require adequate intake of nutrients in order to maintain egg production and offset the energetic costs of reproduction (Alonso-Fernández and Saborido-Rey 2012). Thus, it is not surprising that fathead minnows in RW exposures gained body mass and produced significantly more eggs when fed with a relatively higher food ration. More importantly though, our findings indicate that fish, when supplied with a food ration of 20-30% body weight, were able to sustain normal growth and egg production capacity despite the energetic cost of detoxification and handling of accumulated metals during exposure to the PWE. In contrast, PWE-exposed fish treated with a lower food ration (6-10% of body weight) exhibited a similar decrease in body mass as recorded in RW-fish maintained under an identical diet regime, however the total egg production over 21 days of exposure was significantly lower in the former treatment relative to the latter. This probably occurred as a consequence of the trade off in PWE-exposed fish - the reproductive output was down-regulated in order to offset the energetic cost of metal detoxification and excretion. Although it is to be noted that the energetic cost of metal detoxification and excretion in fish has not yet been quantified in any previous studies, and thus understanding its effect on reproductive performance in fish would require further investigations. Overall, the present study provides strong evidence that the reduced egg production in fathead minnows exposed to 45% PWE, as documented in the previous multi-trophic mesocosms (Rickwood et al. 2006; Rozon-Ramilo et al, 2011a, 2011b), was mediated to a large extent by the decreased food availability. These results provide a probable link between lower invertebrate and fish densities in PWE exposed sites of the Junction Creek watershed (Jaagumagi and Bedard 2002, Sein 1993)

3.4.1 Conclusions

In summary, the findings of the present study indicate that food quantity can play a major role in influencing reproductive capacity of fish during chronic exposure to metal-mine effluents. In addition, it also demonstrates that increased metal concentrations in the diet and/or water during exposure to metal-mine effluent do not necessarily translate into increased tissue metal burden or decreased reproductive output in fish, and these responses can often be profoundly influenced by the nutritional/energetic status of fish. These findings have important implications for evaluating the effects of metal mine effluents on the resident fish populations, since they may experience reduced food abundance in the receiving environment as a consequence of metal mine effluent discharge. In particular, these results suggest that monitoring programs such as the Canadian Environmental Effects Monitoring (EEM) program, which consider responses of invertebrates and fish as a key tool in evaluating environmental health of metal mine effluent exposed sites, should pay particular attention to the implications of indirect effects such as reduced prey abundance to the sustainability of resident fish populations.

4 CHAPTER 4[a]

INFLUENCE OF ELEVATED ALKALINITY AND NATURAL ORGANIC MATTER (NOM) ON TISSUE-SPECIFIC METAL ACCUMULATION AND REPRODUCTIVE PERFORMANCE IN FATHEAD MINNOWS DURING CHRONIC, MULTI-TROPHIC EXPOSURES TO A METAL MINE EFFLUENT

[a] This chapter examines the role of water chemistry (i.e., increased alkalinity and natural organic matter) in influencing toxic responses of fish during chronic exposures to a Canadian metal mine effluent. The purpose of Chapter 4 was to investigate modifying factors of metal toxicity and the role of these factors in mitigating metal bioaccumulation and reproductive impairment in fathead minnows during chronic exposure to the metal mine effluent. Water chemistry was identified in Chapter 2 as a potentially significant factor in influencing toxic responses. This chapter has been accepted in the journal of Ecotoxicology and Environmental Safety (May 17, 2013) under joint authorship with Som Niyogi (University of Saskatchewan) and Monique G. Dubé (Canadian Rivers Institute).

4.1 INTRODUCTION

Metal mines release effluents that can be a significant source of a variety of metals in receiving waterbodies. Although metal concentrations are often elevated in areas that receive metal mine effluents (MMEs), the metals in those ecosystems might not be available or cause toxicity to resident biota. Currently, risk-assessment models that are used to predict metal bioavailability and toxicity in aquatic organisms [e.g., Biotic Ligand Model (BLM)] are mainly focused on assessing toxicity of single metals (Di Toro et al. 2001, Paquin et al. 2000, Santore et al. 2001), and do not include the effects of metal mixtures. The BLM incorporates the influence of water chemistry parameters, such as water hardness, pH, alkalinity, and dissolved organic carbon (DOC), on the availability and binding of free metal ions to the biotic ligand (e.g., fish gill), and predicts toxicity based on the critical accumulation of metal(s) on the biotic ligand (Niyogi and Wood 2004). However, there is a general lack of understanding of how the water chemistry parameters influence metal bioavailability, bioaccumulation and toxicity in chronic conditions, particularly during exposure to complex metal mixtures, such as MMEs.

Water chemistry is likely to influence the toxicity of MMEs to aquatic organisms. In addition to a mixture of metals, MMEs often also contain high levels of various ions (e.g., Ca^{2+}, Mg^{2+} and Na^{+}, and Cl^{-} and SO_4^{-}) (Dubé et al. 2005, Ouellet et al. 2013a, 2013b, Rozon-Ramilo et al. 2011a), which can strongly influence the bioavailability of metals. For example, hardness cations Ca^{2+} and Mg^{2+} can reduce metal bioavailability through competition with free metal ions, whereas natural anions such as Cl^{-} and SO_4^{-} can also decrease metal bioavailability through complexation of free metal ions (Niyogi and Wood, 2004). On the other hand, MMEs, as well as many metal contaminated lakes in the Canadian Shield, generally have relatively low pH/alkalinity (low CO_3^{-}/HCO_3^{-}) and DOC levels (Dubé et al. 2005, Ouellet et al. 2013a, 2013b,

Pyle et al. 2005, Rozon-Ramilo et al. 2011a), which can contribute to increased bioavailability of metals. During acute exposure to single metals, metal complexation associated with increased alkalinity or DOC is known to reduce metal bioavailability and toxicity to aquatic organisms (Pagenkopf et al. 1974, Playle et al. 1993, Schwartz et al. 2004). Similar protective effects of DOC have also been reported in fish exposed to short-term exposures to metal-mixtures (Richards et al. 2001), although the evidence is sporadic. Importantly though, the water chemistry parameters, which are known to reduce acute metal toxicity, have been reported to be either less effective or ineffective during chronic exposure to metal(s) (Brauner and Wood 2002a, 2002b, De Schamphelaere and Janssen 2004a, 2004b). Moreover, it is largely unknown whether water chemistry parameters can influence metal bioavailability and toxicity to fish during chronic exposure to metal mixtures. Recent evidence indicates no apparent interrelationship among the free metal ion activity in the exposure water, and bioaccumulation and chronic toxicity in fish exposed to metal mixtures (Kamunde and MacPhail 2011). Exposure to metals in mixture is likely to elicit competitive or additive interactions, considerations that are not included in the current BLM, and therefore limit its ability to predict toxicity of metals in contaminated aquatic ecosystems, where organisms are almost always exposed to metals in mixture (Borgmann et al. 2008). In addition, fish inhabiting the metal-contaminated environments are exposed to metal mixtures via both water and diet (Wang 2011), and the latter pathway is not included in the current BLMs.

It is apparent that further investigations are needed to better understand the influence of water chemistry on the bioavailability of metals to fish during chronic exposure to MMEs. This is important since MMEs have been found to elicit a variety of toxic effects to fish in both field-based and laboratory studies, and the effects included delayed development and larval

deformities (Driessnack et al. 2011, Jezierska et al. 2009), decreased lipid storage and growth (Bennett and Janz 2007), as well as decreased fecundity (Driessnack et al. 2011, Franssen 2009, Rozon-Ramilo et al. 2011a, 2011b), and behavioural changes (Gerhardt 1998, McPherson et al. 2004). Generally, the effects observed in fish exposed to MMEs are believed to be induced by the accumulation of metals in target organs (e.g., gill, liver and gonad), however a causal relationship between metal bioaccumulation and toxicity has not been clearly established in any previous studies.

The main objective of this study was to examine whether increased alkalinity (achieved by adding $NaHCO_3$) or natural organic matter (NOM) (added as humic acid) ameliorate tissue-specific metal accumulation and reproductive toxicity in a model fish species, the fathead minnow (*Pimephales promelas*), during environmentally relevant chronic exposure to a MME. Since both alkalinity and NOM are believed to reduce metal bioavailability by increased complexation of free metals in the exposure water, we hypothesized that increased $NaHCO_3$ or humic acid content would cause a decrease of metal accumulation in target organs and protect against the MME-induced reproductive impairment in fathead minnows. The MME used in the present study was process water effluent (PWE), which has been found to cause metal accumulation in benthic invertebrate and fish tissues, and impair reproductive output of fish in a number of previous studies (Ouellet et al. 2013a, 2013b, Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a, 2011b). PWE is released at concentrations of approximately 51,000,000 m^3/year (2009) into the Junction Creek Watershed, near Sudbury, Ontario, Canada, and is diluted to a concentration of approximately 45% in the receiving environment, based on discharge and stream conditions (Rickwood et al. 2006a).

4.2 MATERIALS & METHODS

4.2.1 Multi-trophic mesocosms

This research was performed at the University of Saskatchewan in Saskatoon, SK, Canada, from January to March 2011. Multi-trophic mesocosms with 6 replicates (10.3-L circular polyethylene streams) were used to mimic natural stream systems. Detailed descriptions of the multi-trophic systems can be found in Rickwood et al. (2006a) and Hruska and Dubé (2004). Each replicate stream was made up of a sediment layer (~2.5 cm of pre-cleaned silica sand), a feeding barrier for providing consistent numbers of *Chironomus dilutus* as food to fathead minnows during exposure, a spawning tile, and a mesh-screen for preventing adult *C. dilutus* and fathead minnows from escaping the streams. Water was exchanged at a rate of 1 turnover/day into an 85-L reservoir, which evenly recirculated the treatment water to each of the mesocosm streams via a March pump (Model LC-3CP-MD, March Manufacturing, Glenview, IL, USA). Streams were aerated with air-stones and heated to 25 ± 2°C with submersible aquarium heaters under conditions of 16h light:8h dark photoperiod. The mesocosm system allowed for both water-borne and dietary exposures to the MME, where daily egg production and tissue-specific metal accumulation in fathead minnows were primary endpoints. The bioaccumulation of metals from MMEs to target organisms and tissues (i.e., *C. dilutus*, fathead minnow liver, gonad, gill, and carcass tissues) have been evaluated using these mesocosms in many regions, including Ontario (Dubé et al. 2006, Hruska and Dubé 2004, 2005, Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a, 2011b), New Brunswick (Dubé et al. 2005), Saskatchewan (Driessnack et al. 2011), and the Northwest Territories (Spencer et al. 2008). The 21-day multi-trophic mesocosms used in our study are an accepted component of Environment Canada's Environmental Effects

Monitoring (EEM) program. Animal use was approved by the University of Saskatchewan Committee on Animal Care and Supply (UCACS) and Animal Research Ethics Board (AREB).

4.2.2 Process water effluent and reference water

The effluent used in this study was the process water effluent (PWE) from an Ontario metal mine (Vale Canada Limited). This effluent was chosen because it is discharged at high volumes (~51,000,000 m^3/year in 2009 in the Junction Creek watershed) and has been shown to cause significant decreases in cumulative egg production of fathead minnows in several studies (Ouellet et al. 2013a, 2013b, Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a, 2011b). Process water effluent exposures were performed at a concentration of 45% dilution based on environmentally relevant concentrations in the Junction Creek stream watershed. Effluent was shipped weekly from Sudbury, Ontario to the University of Saskatchewan. For the present study, reference water (RW) was made up of a mixture of reverse osmosis (RO) water and dechlorinated laboratory water at concentrations of approximately 65% RO and 35% laboratory water in order to match the water chemistry conditions (pH, alkalinity, hardness, background metal concentrations) of a previously used reference river site (Vermillion River), located near the Junction Creek Watershed (Rozon-Ramilo et al. 2011a). The same reference water was also used as dilution water for the 45% PWE.

4.2.3 Treatments

This study consisted of six treatment groups (see Table 1): i) Reference Water (RW), ii) RW + increased alkalinity, iii) RW + increased DOC, iv) 45% PWE, v) 45% PWE + increased alkalinity, and vi) 45% PWE + increased DOC. In order to increase the alkalinity of the respective RW or 45% PWE treatments, sodium bicarbonate ($NaHCO_3$; 99%) (Alfa-aesar, Heysham, Lancs, UK, 99%) was added until a pH of 8.1 was achieved. Humic acid (as sodium

salt) (Alfa-aesar, Ward Hill, MO, USA, 50-60%) was added to the respective RW or 45% PWE treatments in order to increase the DOC by a nominal concentration of 3 mg/L. We chose a modest increase in DOC level in our exposures because of the low solubility of commercial humic acid used in our experiment. Depending on the treatment, $NaHCO_3$ or humic acid was added to RW or 45% PWE in 330L polyethylene holding tanks, and allowed to reach chemical equilibrium over 24 hrs. During equilibration, the exposure waters were stirred constantly with a Stir-Pak® mixer (Cole-Parmer, Montreal, QC, Canada) to facilitate solubility. Subsequently, the respective exposure water was pumped (Pulsatron Series E, Viking Pump of Canada, Edmonton, AB, Canada) into the multi-trophic mesocosms.

4.2.4 Trophic-transfer system

Chironomus dilutus larvae were cultured directly in each of the six reference and treatment streams in order to provide a dietary exposure to fathead minnows in addition to the waterborne exposure. Egg sacs of *C. dilutus* were isolated from laboratory held brood stocks every 7 days for 3 weeks, and added directly to the replicate streams to provide two eggs sacs per stream (equivalent to 1 g/day of food for each pair of fish for the duration of the 21-day exposure period) (Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a). During the *C. dilutus* culturing period, water exchanges were performed at a rate of ½ exchanges every second day, except during the first week of culturing when the first water exchange was carried out on day 4, to minimize disturbances while *C. dilutus* cultures were being established.

4.2.5 Pre-exposure period

The pre-exposure period was performed in order to ensure all pairs of fathead minnows selected for the exposure period were capable spawners, as well as to provide similar reproductive potential for each replicate stream. Six to nine-month old fathead minnows were

obtained from Osage Catfisheries Inc. (Osage Beach, MO, USA). On day-0 of the pre-exposure, body weight, total length, and secondary sex characteristics (banding, nuptial tubercles, dorsal pad, fin dot, and ovipositor) were recorded for each fathead minnow (83 pairs). Fathead minnows were placed randomly into each stream until one male and one female were present in each (one breeding pair/stream). Water exchanges were fixed at one turnover/day. Breeding pairs were fed frozen bloodworms (Sally's bloodworms^{TM}, San Francisco Bay Brand, Inc., Newark, CA, USA) twice daily at a feeding rate of approximately 1 gram/day. Egg production was monitored daily for seven days by removing the breeding tile from each stream, scraping eggs onto a petri dish, and photographing the eggs with a Canon Powershot A620 digital camera mounted to a Vista Vision^{TM} (Model 48402-00, VWR International, Missisauga ON, Canada). Breeding pairs that spawned at least once in the pre-exposure period with >80% fertilization success were used in the exposure period. This consisted of thirty-six pairs being distributed among the six treatments (n=6 for each treatment). Infertile eggs were either opaque, had a visibly precipitated yolk, or contained no yolk (Ankley et al. 2001). One-way ANOVA was performed on the pre-exposure egg production (total number of eggs, eggs/female/day, and breeding attempts) to verify that there were no significant differences between egg production for fathead minnows in each of the treatments (α=0.05, n=6) (see Appendix Figure A-1 for mean daily egg production). Breeding pairs were distributed randomly to a stream within the treatment.

4.2.6 Exposure period

Fathead minnows were exposed to the treatments for 21 days. Daily water quality measurements were performed at the University of Saskatchewan. Temperature, dissolved oxygen (DO), conductivity [YSI meter (Yellow Springs Instruments, Yellow Springs, OH,

USA)], ammonia (Rolf C. Hagen, Edmonton, AB, Canada), pH (Oakton pHTestr, Oakton Instruments, Vernon, IL, USA), and alkalinity (LaMotte Company, Chestertown, MD, USA) were each measured from one stream, randomly, per treatment. Weekly water samples were taken on days 7, 14, and 21 from each treatment, randomly, in pre-labeled high density polyethylene (HDPE) sample bottles. Samples were taken directly from a single stream which was chosen randomly from each mesocosm table, filtered into the sample bottles with a 0.45 µm cellulose acetate (CA) filter (Corning Incorporated, Corning, NY, USA), sealed in a ziplock bag, and shipped out to Testmark Laboratories (Sudbury, Ontario, Canada) in a cooler chilled with ice for further analysis. These water samples were analyzed for dissolved metals using inductively coupled plasma-mass spectrometry (ICP-MS). Similarly, Ca^{2+} and Mg^{2+} concentrations were also measured using ICP-MS without acidification, and converted to total water hardness [expressed as the equivalent of calcium carbonate ($CaCO_3$)]. In addition, anions were analyzed by Ion Chromatography, and dissolved organic carbon (DOC) was measured using a Dohrman total organic carbon (TOC) analyzer. Minimum detection limits for DOC and TOC were 0.4 mg/L. All of these water quality measurements were conducted by Testmark Laboratories, following the analytical methodology of the American Public Health Association (APHA) and US Environmental Protection Agency (EPA).

During the exposure period, egg production, egg sizes, fathead minnow larvae, and *C. dilutus* emergence were monitored daily. Each day, breeding tiles were checked for eggs, and eggs were scraped onto a petri dish and photographed. Ten eggs per brood were selected and analyzed with Image Pro Plus 6.1 (Media Cybernetics Inc., Maryland, USA) for egg size determinations. Subsequently, the eggs were placed into egg cups, returned to the respective treatment, and aerated. Unfertilized eggs and dead eggs were counted and removed daily until

hatching was complete. Once hatched, larvae were moved into petri dishes and photographed with the microscope to assess larval deformities. At the end of the exposure period, fathead minnows were anesthetized with tricaine methanesulfonate (MS-222, Sigma-Aldrich, St Louis, MO, USA), assessed for secondary sex characteristics, total body weight, total length, and dissected to obtain livers, gills, gonads, and the carcass. On the final day of the exposure period, three 9-cm^2 cores per stream were taken to determine densities of *C. dilutus* larvae. The number of 3^{rd} and 4^{th} instar *C. dilutus* was counted from the three cores to determine an average number of *C. dilutus* per stream. One gram (wet weight) of *C. dilutus* were also collected from three randomly selected streams per treatment and stored in a cooler of dry ice along with the fish tissue samples, and shipped out to Testmark Laboratories for metal analyses. Metal analysis in the fish tissues and *C. dilutus* was carried out using ICP-MS following microwave digestion. In order to maintain quality control and quality assurance of metal analysis, Testmark Laboratories used method blanks, positive controls and, blank spikes, and also analyzed a certified reference material [DOLT-3 dogfish (*Squalus acanthias*) liver, National Research Council of Canada]. Percentage recovery for the elements analyzed ranged from 84.06 to 111.04%.

4.2.7 Metal speciation analysis

We used the geochemical speciation model, Visual MINTEQ, version 3.0 (KTH, Department of Land and Water Resources Engineering, Stockholm, Sweden), to estimate the free ion concentrations of various metals in the 45% PWE treatments. The mean values presented in Tables 4-1 and 4-2 were used to run the speciation modeling. The dissolved organic carbon content of each treatment was assumed to be 60% humic acid and 40% fulvic acid (Kamunde and MacPhail 2011).

4.2.8 Exposure analysis and statistics

Data were analyzed and graphed using IBM SPSS Statistics 20.0.0 (SPSS, Chicago, IL) and Sigmaplot® Version 11.0 (San Jose, CA, USA). Water chemistry, and metal burdens in *C. dilutus* and fish tissues were analyzed using one-way analysis of variance (ANOVAs). Adult survival, condition factor (k), liver somatic index [LSI (%)], and gonadal somatic index [GSI (%)], as well as mean egg sizes, mean total deformities (%), mean fertilization success (%), and mean densities of *C. dilutus* per replicate were also analyzed using one-way ANOVAs. The Shapiro-Wilk test was used to test parametric assumptions for normality and Levine's test was used to test for homogeneity over variance prior to the one-way ANOVA analysis. Data that failed these assumptions were either transformed [arcsin(%) or $\log_{10}$] to achieve normal distribution or analyzed using the non-parametric Kruskal-Wallis test. If significant differences were detected by ANOVA, Tukey's post-hoc test was used to determine the differences between the treatment and the reference(s) and/or among the treatments. Cumulative mean daily egg production (calculated as: # eggs/breeding pair each day, summed cumulatively for 21 days) and cumulative total spawning events were compared between each treatment by performing multiple, two-sample Kolmogorov-Smirnov tests.

4.3 RESULTS

4.3.1 Water Chemistry

4.3.1.1 RW+increased alkalinity and RW+increased DOC *vs.* RW

Alkalinity, pH, conductivity, and sodium concentrations were all significantly greater in the RW+increased alkalinity treatment relative to the other RW treatments, while DOC was significantly greater (~60% increase) in the RW+increased DOC treatment relative to the other RW treatments (Tukey's HSD post hoc test; $p<0.05$) (Table 4-1). The measured DOC

Table 4-1 – Summary of general water quality measurements from unmodified and modified (increased alkalinity and increased DOC) reference water (RW) and 45% process water effluent (PWE) treatments

Parameter							
General WQ		**RW**	**RW+inc. alk.**	**RW+inc. DOC**	**45%PWE**	**45%PWE+inc. alk.**	**45%PWE+inc. DOC**
Alkalinity	mg/L	32.2±0.8^{ab}	84.4±3.6^{c}	37.0±1.4^{b}	23.6±2.5^{ae}	103.8±4.3^{d}	19.8±2.9^{e}
pH	pH	7.7±0.0^{a} (7.6)	8.1±0.0^{b} (8.1)	7.8±0.0^{a} (7.6)	7.2±0.1^{c} (6.9)	8.1±0.0^{b} (8.1)	7.1±0.1^{c} (6.9)
DOC*	mg/L	4.0±0.2^{a} (3.0)	4.0±0.4^{a} (3.0)	6.4±0.1^{b} (6.0)	5.0±0.3^{a} (3.0)	4.9±0.2^{a} (3.0)	6.2±0.2^{b} (6.0)
Temperature	°C	23.9±0.0^{a}	24.1±0.0^{a}	24.7±0.0^{a}	24.4±0.2^{a}	23.8±0.2^{a}	24.3±0.1^{a}
Ammonia	mg/L	0.16±0.10^{a}	0.80±0.27^{ab}	0.89±0.27^{ab}	2.70±0.51^{c}	1.74±0.47^{bc}	1.08±0.44^{abc}
Chloride	mg/L	3.07±0.31^{a}	3.26±0.39^{a}	4.73±1.69^{a}	37.87±0.41^{b}	37.87±0.32^{b}	36.67±0.50^{b}
Conductivity	mg/L	146.6±2.6^{a}	234.5±5.5^{b}	154.7±2.7^{a}	1590.0±16.8^{c}	1617.4±15.0^{c}	1579.7±8.1^{c}
Dissolved Oxygen	%	85.1±0.7^{a}	84.0±1.0^{a}	84.4±0.9^{a}	84.1±0.8^{a}	85.3±1.0^{a}	85.4±0.8^{a}
Nitrate	mg/L	0.45±0.33	0.19±0.03^{a}	0.24±0.12^{a}	0.50±0.00^{a}	0.50±0.00^{a}	0.50±0.00^{a}
Sodium	mg/L	9.64±0.47^{a}	32.23±0.92^{b}	10.37±0.72^{a}	64.17±1.74^{c}	113.67±7.51^{d}	67.07±2.07^{c}
Sulfate	mg/L	25.03±4.91^{a}	26.93±1.98^{a}	26.30±3.41^{a}	663.00±44.84^{b}	665.00±21.28^{b}	644.67±48.20^{b}
Total Hardness (as $CaCO_3$)	mg/L	49.6±6.3^{a}	48.4±3.1^{a}	43.7±4.1^{a}	700.3±23.2^{b}	644.7±43.1^{b}	707.7±49.15^{b}
Calcium	mg/L	12.85±2.53^{a}	12.15±1.52^{a}	10.93±1.79^{a}	237.33±10.81^{b}	214.67±15.45^{b}	241.67±21.28^{b}
Magnesium	mg/L	4.26±0.14^{a}	4.41±0.43^{a}	4.00±0.32^{a}	26.13±1.34^{b}	26.37±1.43^{b}	25.23±1.52^{b}

Data is presented as mean±S.E.; n=21 for pH, alkalinity, temperature, ammonia, conductivity, and dissolved oxygen and n=3 for all remaining parameters
Data in parentheses represent nominal values
Means that do not share letters are statistically different from each other (one-way ANOVA; Tukey HSD post hoc test, $p<0.05$)
*dissolved organic carbon

concentration was similar to nominal concentrations in RW+increased DOC. There were no significant differences in dissolved metal concentrations among any of the RW treatments (ANOVA; $p>0.05$) (Table 4-2).

4.3.1.2 45% PWE *vs.* RW

The pH was significantly lower in 45% PWE relative to RW (Tukey's HSD post hoc test; $p<0.05$), while ammonia, chloride, conductivity, nitrate, sodium, sulfate, total hardness, calcium, and magnesium measurements were significantly greater in the 45% PWE treatment relative to the RW treatment (Tukey's HSD post hoc test; $p<0.05$) (Table 4-1). The 45% PWE exposure water contained elevated levels of eleven metals (barium, boron, cobalt, copper, lithium, manganese, molybdenum, nickel, rubidium, selenium, and strontium) relative to the RW exposure (Tukey's HSD post hoc test; $p<0.05$ for each metal) (Table 4-2).

4.3.1.3 45% PWE+increased alkalinity and 45% PWE+increased DOC *vs.* 45% PWE

The alkalinity increased by more than four-fold in the 45% PWE+increased alkalinity treatment relative to the other 45% PWE treatments, and both pH, and sodium concentrations were significantly greater as well (Tukey's HSD post hoc test; $p<0.05$). Similarly, DOC was significantly greater (~25% increase) in the 45% PWE+increased DOC treatment relative to the other 45% PWE treatments (Tukey's HSD post hoc test; $p<0.05$) (Table 4-1). Dissolved metal concentrations were mostly similar among 45% PWE treatments, except for concentrations of manganese, which were significantly lower in the 45% PWE+increased alkalinity treatment relative to the other 45% PWE treatments (Tukey's HSD post hoc test; $p<0.05$), and concentrations of copper, which were significantly lower in the 45% PWE+increased DOC treatment relative to the other 45% PWE treatments (Tukey's HSD post hoc test; $p\leq0.002$) (Table 4-2).

Table 4-2 – Summary of dissolved metal concentrations present in unmodified and modified (increased alkalinity and increased DOC) reference water (RW) and 45% process water effluent (PWE) treatments

Parameter							
Element		**RW**	**RW+inc. alk.**	**RW+inc. DOC**	**45% PWE**	**45%PWE+inc. alk.**	**45%PWE+inc. DOC**
Barium	µg/L	7.73±0.41^{a}	9.90±2.08^{a}	7.57±0.55^{a}	29.93±1.30^{b}	26.40±0.97^{b}	30.37±1.29^{b}
Boron	µg/L	23.73±0.78^{a}	23.90±1.25^{a}	23.43±2.09^{a}	69.30±1.17^{b}	59.97±2.85^{b}	68.47±3.24^{b}
Cadmium	µg/L	0.28±0.23^{a}	0.07±0.02^{a}	≤0.05±0.00^{a}	0.19±0.04^{a}	0.07±0.02^{a}	0.13±0.01^{a}
Cobalt	µg/L	≤0.50±0.00^{a}	≤0.50±0.00^{a}	≤0.50±0.00^{a}	3.45±0.76^{b}	2.19±0.10^{b}	3.08±0.61^{b}
Copper	µg/L	11.20±1.87^{a}	9.60±1.82^{a}	6.83±2.74^{a}	56.87±3.79^{b}	49.40±6.07^{b}	23.00±2.25^{c}
Lithium	µg/L	4.43±0.99^{a}	3.47±0.97^{a}	3.40±0.90^{a}	30.67±1.76^{b}	28.00±2.00^{b}	32.67±1.86^{b}
Manganese	µg/L	0.93±0.43^{a}	1.43±0.93^{a}	0.83±0.33^{a}	30.20±12.88^{b}	4.37±0.32^{ab}	25.33±11.87^{b}
Molybdenum	µg/L	≤0.50±0.00^{a}	≤0.50±0.00^{a}	≤0.50±0.00^{a}	3.67±0.38^{b}	3.13±0.12^{b}	3.53±0.23^{b}
Nickel	µg/L	2.53±0.33^{a}	1.90±0.15^{a}	2.40±0.10^{a}	77.33±5.88^{b}	61.67±3.84^{b}	80.27±10.18^{b}
Rubidium	µg/L	≤0.50±0.00^{a}	≤0.50±0.00^{a}	0.70±0.20^{a}	31.37±1.35^{bc}	29.17±0.76^{b}	32.67±0.44^{c}
Selenium	µg/L	≤0.50±0.00^{a}	≤0.50±0.00^{a}	≤0.50±0.00^{a}	7.27±0.48^{b}	6.67±0.38^{b}	8.23±0.58^{b}
Strontium	µg/L	80.87±12.88^{a}	77.60±7.58^{a}	71.60±10.20^{a}	600.67±22.98^{b}	541.00±41.33^{b}	598.00±46.92^{b}
Thallium	µg/L	≤0.05±0.00^{a}	≤0.05±0.00^{a}	≤0.05±0.00^{a}	0.08±0.03^{a}	0.09±0.02^{a}	0.09±0.02^{a}
Zinc	µg/L	14.13±5.36^{a}	20.00±8.89^{a}	15.00±6.34^{a}	20.33±5.78^{a}	13.33±3.61^{a}	18.40±4.46^{a}

Data is presented as mean±S.E.; n=3 for all parameters

Means that do not share letters are statistically different from each other (one-way ANOVA; Tukey HSD post hoc test, $p<0.05$)

4.3.2 Predicted proportions of free metal ions in 45% PWE+increased alkalinity and 45% PWE+increased DOC vs. 45% PWE

In general, the proportion of free ions for all of the metals examined were >40% in each of the 45% PWE treatments, except for copper (<3%) (Table 4-3). Increased alkalinity in 45% PWE contributed to a predicted increase in carbonate-bound proportion of cobalt (~6.5% increase), manganese (~11% increase), nickel (~11% increase), and zinc (~10% increase) (data not shown), which also coincided with a decrease (7-20%) in predicted free ion concentrations of the same metals (Table 4-3). Notably, free copper ion concentration reduced to an almost negligible proportion (0.13%) in the 45% PWE+increased alkalinity treatment (Table 4-3). In contrast, increased DOC level in the 45%PWE did not affect the free ion concentration of any metals except copper, which showed a ~70% decrease relative to that in the unmodified 45% PWE treatment (Table 4-3). Due to the high sulfate level in the 45% PWE, the predicted proportion of sulfate-bound metals in each of the 45% PWE treatments was relatively high. The sulphate species of barium, cobalt, manganese, nickel, strontium, and zinc were >30% for each of these metals, while the proportion of sulphate-bound rubidium and thallium were ~5% and ~16%, respectively (data not shown).

4.3.3 Metal burdens of Chironomus dilutus

There were no significant differences in the metal concentrations of *C. dilutus* larvae among RW treatments (Tukey's HSD post hoc test; $p>0.05$). Although eleven metals were found to be at elevated levels in the 45% PWE exposure water relative to the reference water, only six metals (cobalt, copper, nickel, rubidium, selenium and thallium) were present at significantly elevated levels in *C. dilutus* larvae in the 45% PWE treatments relative to the RW

Table 4-3 – Proportions (%) of free metal ions from the unmodified and modified (increased alkalinity and increased DOC) 45% process water effluent (PWE) treatments determined from Visual MINTEQ speciation modeling

Parameter				
Metal		**45% PWE**	**45%PWE+ inc. alk.**	**45%PWE+ inc. DOC**
Barium	Free metals (%)	66.17	65.90	66.64
Cobalt	Free metals (%)	56.14	52.08	56.60
Copper	Free metals (%)	2.34	0.13	0.72
Lithium	Free metals (%)	96.52	96.50	96.61
Manganese	Free metals (%)	59.58	53.12	60.18
Nickel	Free metals (%)	54.36	48.06	54.26
Rubidium	Free metals (%)	93.35	93.32	93.52
Strontium	Free metals (%)	57.08	56.93	57.64
Thallium	Free metals (%)	83.72	83.55	84.07
Zinc	Free metals (%)	49.93	39.99	50.18

treatments (Tukey's HSD post hoc test; $p<0.05$) (Figure 4-1). There were, however, some differences in the tissue metal concentrations of *C. dilutus* larvae among 45% PWE treatments. Concentrations of cobalt and nickel in *C. dilutus* larvae were approximately two-fold greater in the 45% PWE+increased alkalinity treatment relative to the 45% PWE treatment (Tukey's HSD post hoc test; $p=0.002$ and $p=0.012$, respectively). Similarly, concentrations of copper in *C. dilutus* larvae were ~50% greater in the 45% PWE+increased DOC treatment relative to the unmodified 45% PWE treatment (Tukey's HSD post hoc test; $p=0.036$).

4.3.4 Fathead minnow metal body burdens

In general, there were no significant differences in the concentration of any metals in any tissues examined among female fathead minnows from the RW treatments (Tukey's HSD post hoc test; $p>0.05$). There were, however, significant differences in tissue-specific metal concentrations in female fathead minnows exposed to 45% PWE relative to RW treatments (Figure 4-2). The tissue concentrations of rubidium (liver, gonads, gills, and carcass), selenium (liver, gonads, and gills), nickel (gonads and gills), thallium (gonad and carcass), and copper (gills) were significantly elevated in fish exposed to 45% PWE relative to RW (Tukey's HSD post hoc test; $p<0.05$). In addition, the tissue-specific metal concentrations in fish were similar among all three 45% PWE treatments except for copper. The gill copper concentration was significantly greater in fish exposed to the 45% PWE with increased DOC relative to that in the unmodified 45% PWE treatment (Tukey's HSD post hoc test; $p=0.033$).

4.3.5 Biological endpoints and fecundity

The survival of adult fish was not impacted by any of the treatments (ANOVA; $p>0.05$) (data not shown). Similarly, there were no statistically significant differences in GSI (%), LSI (%), or condition factor for either females or males among the six different treatments (ANOVA;

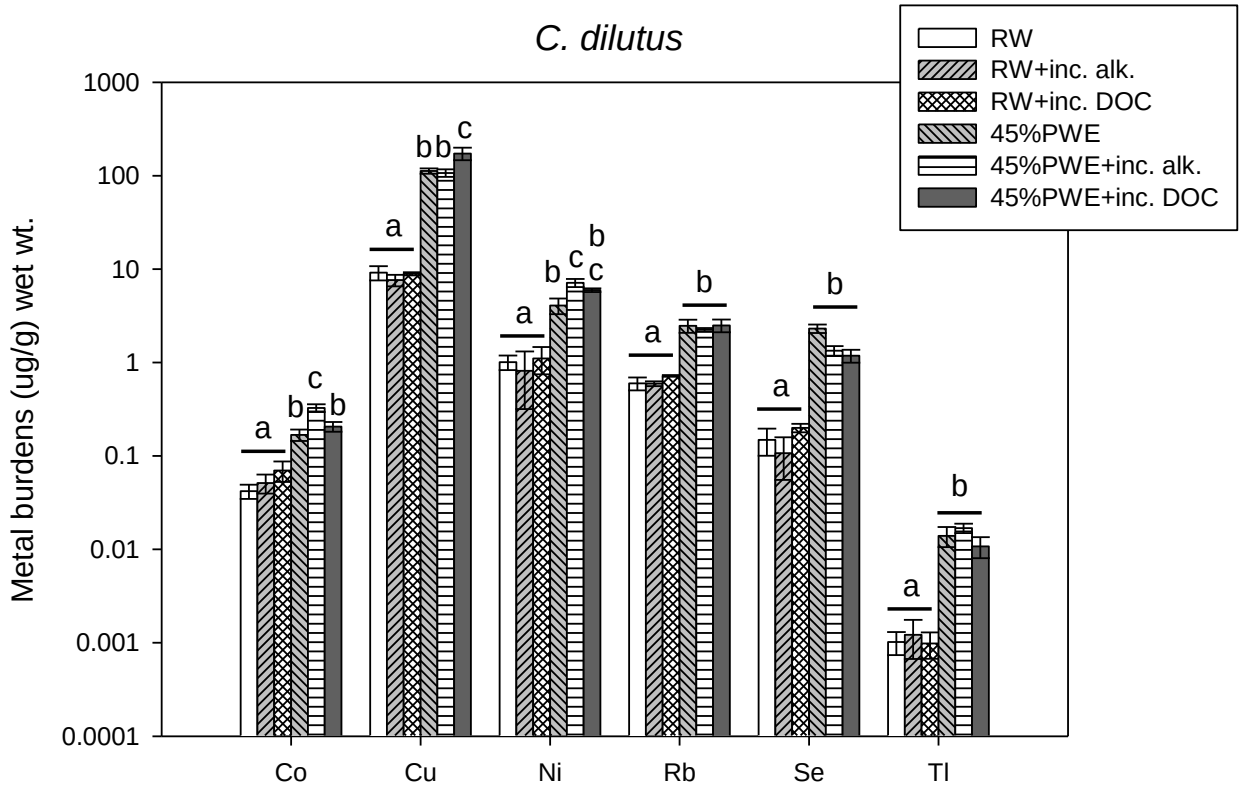

Figure 4-1 – Mean concentrations of metals (±S.E.) that were elevated in *C. dilutus* larvae exposed to 45% process water effluent (PWE). Letters indicate significant differences, where means that do not share letters are statistically different from one another for that metal (one-way ANOVA; Tukey HSD post hoc test, $p<0.05$).

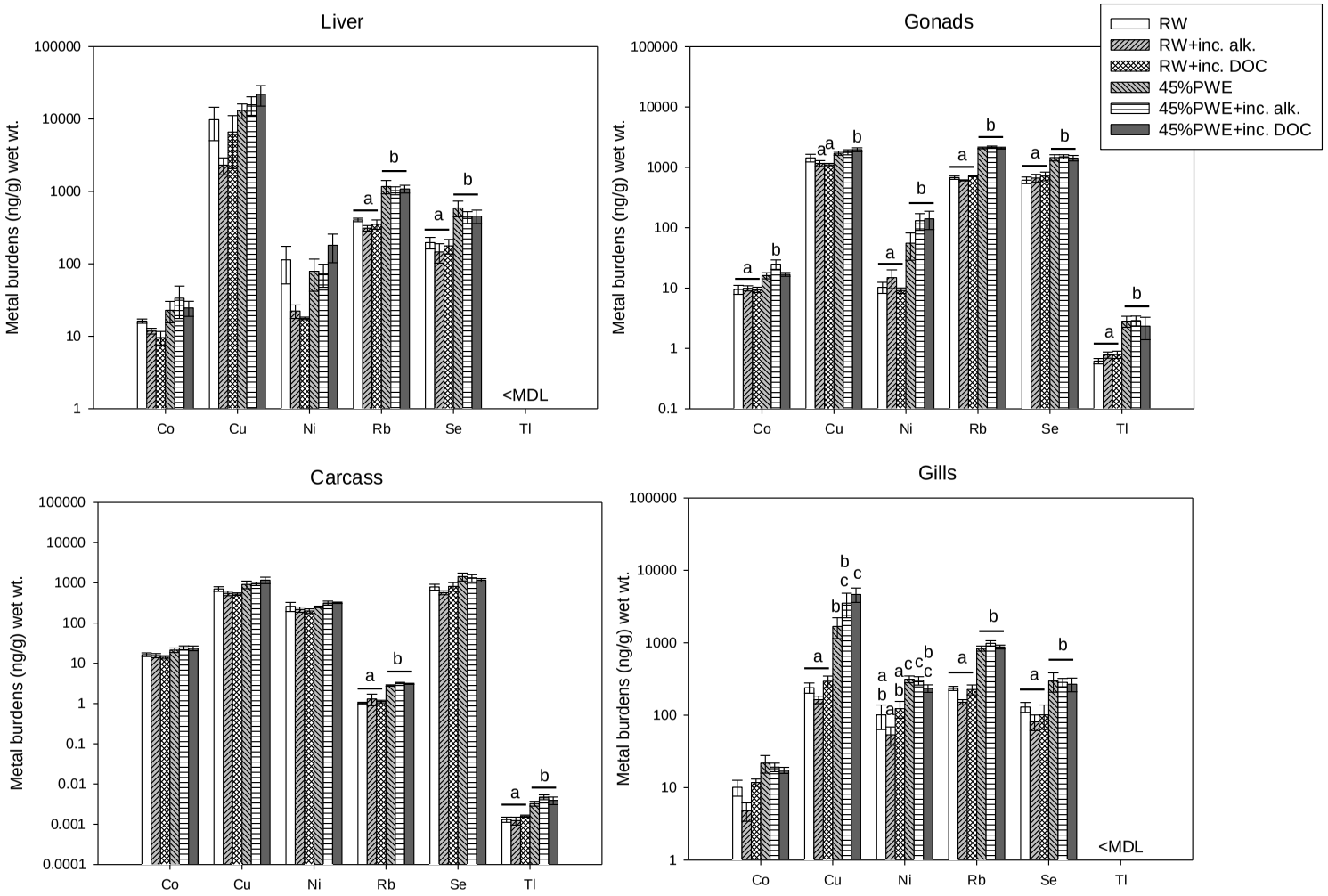

Figure 4-2 – Mean concentrations of metals (±S.E.) that were elevated in various female fathead minnow liver, gonads, carcass, and gill tissues exposed to 45% process water effluent (PWE). Letters indicate significant differences, where means that do not share letters are statistically different from one another for that metal and tissue (one-way ANOVA; Tukey HSD post hoc test, $p<0.05$). <MDL indicates less than minimum detection limits.

p>0.05) (Table 4-4, Table 4-5). There were also no statistically significant differences observed in hatch rate, larval deformities, or egg sizes among any of the six different treatments (ANOVA; p>0.05 for each) (see Appendix Figure A-2 for rate of larval deformities, Appendix Figure A-3 for hatch rate, and Appendix Figure A-4 for mean egg sizes).

Fathead minnow egg production, measured as cumulative mean daily egg production, was variable among the treatments over the 21-day exposure period (Figure 4-3). Fish in the RW+increased alkalinity and RW+increased DOC treatments produced significantly more eggs (+496.0 eggs/pair and +588.6 eggs/pair, respectively) relative to fish in the RW treatment (two-sample Kolmogorov-Smirnov test; p=0.002 and p=0.001, respectively). In contrast, fish in the 45% PWE treatment produced significantly fewer eggs than fish in the RW treatment (-301.8 eggs/pair) (two-sample Kolmogorov-Smirnov test; p=0.002). Interestingly, similar to our observation in the RW treatments, fish exposed to 45% PWE either with elevated alkalinity or elevated DOC level produced significantly more eggs than fish in the 45% PWE treatment (+569.8 eggs/pair and +261.5 eggs/pair, respectively) (two-sample Kolmogorov-Smirnov test; p≤0.001 for both). Consistent to this observation, we also recorded a trend towards greater brood sizes in fish in the modified treatments relative to the unmodified treatments irrespective of 45% PWE exposure, although this difference was not statistically significant (Kruskal-Wallis; p=0.102) (Figure 4-4).

4.3.6 Densities of Chironomus dilutus

Densities of *C. dilutus* measured on day 21 of the exposure period are presented in Figure 4-5. The highest density of *C. dilutus* larvae (1.35 larvae/cm^2) was observed in the RW treatment, followed closely by the RW+increased DOC treatment (1.11 larvae/cm^2). The RW and RW+increased DOC treatments were the only two treatments that contained approximately

Table 4-4 – Gonadosomatic index (GSI), liver somatic index (LSI), and condition factor (K) for female fathead minnows (*P. promelas*) from unmodified and modified (increased alkalinity and increased DOC) reference water (RW) and 45% process water effluent (PWE) treatments

Treatment	n	GSI (%)	LSI (%)	K
RW	5	16.2±1.8	2.6±0.4	1.1±0.0
RW+inc. alk.	6	12.2±1.2	2.5±0.3	1.0±0.1
RW+inc. DOC	5	13.4±2.6	2.2±0.3	1.0±0.1
45%PWE	6	10.6±2.2	2.5±0.3	1.0±0.0
45%PWE+inc. alk.	6	13.8±1.2	2.5±0.3	1.0±0.0
45%PWE+inc. DOC	5	12.7±1.3	2.5±0.3	0.9±0.1

Data are presented as mean ± S.E.

Table 4-5 – Gonadosomatic index (GSI), liver somatic index (LSI), and condition factor (K) for male fathead minnows (*P. promelas*) from unmodified and modified (increased alkalinity and increased DOC) reference water (RW) and 45% process water effluent (PWE) treatments

Treatment	n	GSI (%)	LSI (%)	K
RW	5	0.8±0.2	2.2±0.2	1.1±0.1
RW+inc. alk.	6	1.2±0.1	1.3±0.3	1.1±0.0
RW+inc. DOC	4	0.9±0.1	2.1±0.4	1.1±0.0
45%PWE	5	1.1±0.1	1.7±0.3	1.1±0.1
45%PWE+inc. alk.	6	1.1±0.2	1.7±0.2	1.1±0.1
45%PWE+inc. DOC	5	0.9±0.2	1.8±0.4	1.1±0.0

Data are presented as mean ± S.E.

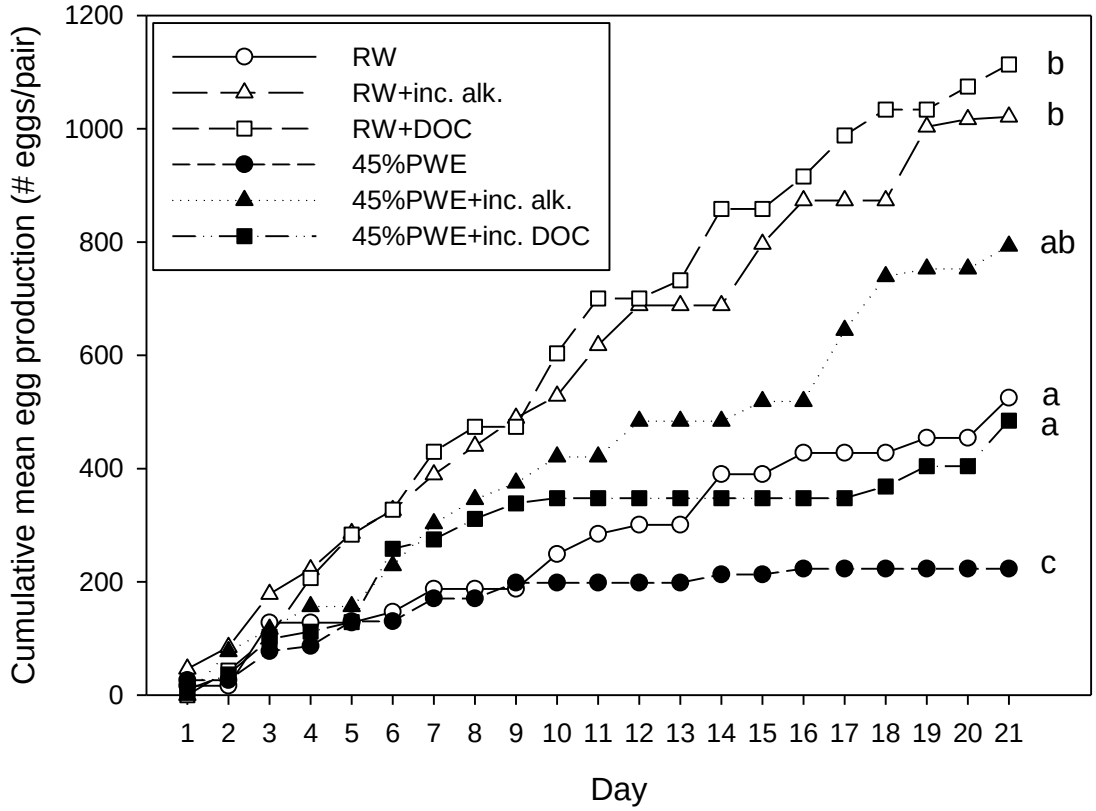

Figure 4-3 – Cumulative daily mean egg production (measured as eggs/pair) for breeding pairs of fathead minnows (*P. promelas*) over the 21-day exposure period in unmodified and modified (increased alkalinity and increased DOC) reference water (RW) and 45% process water effluent (PWE) treatments. White symbols represent reference water (RW) treatments and black symbols represent 45% process water effluent (PWE) treatments. Treatment groups that do not share letters are statistically different from each other (two-sample Kolmogorov-Smirnov test; $p<0.05$ for all pairwise comparisons).

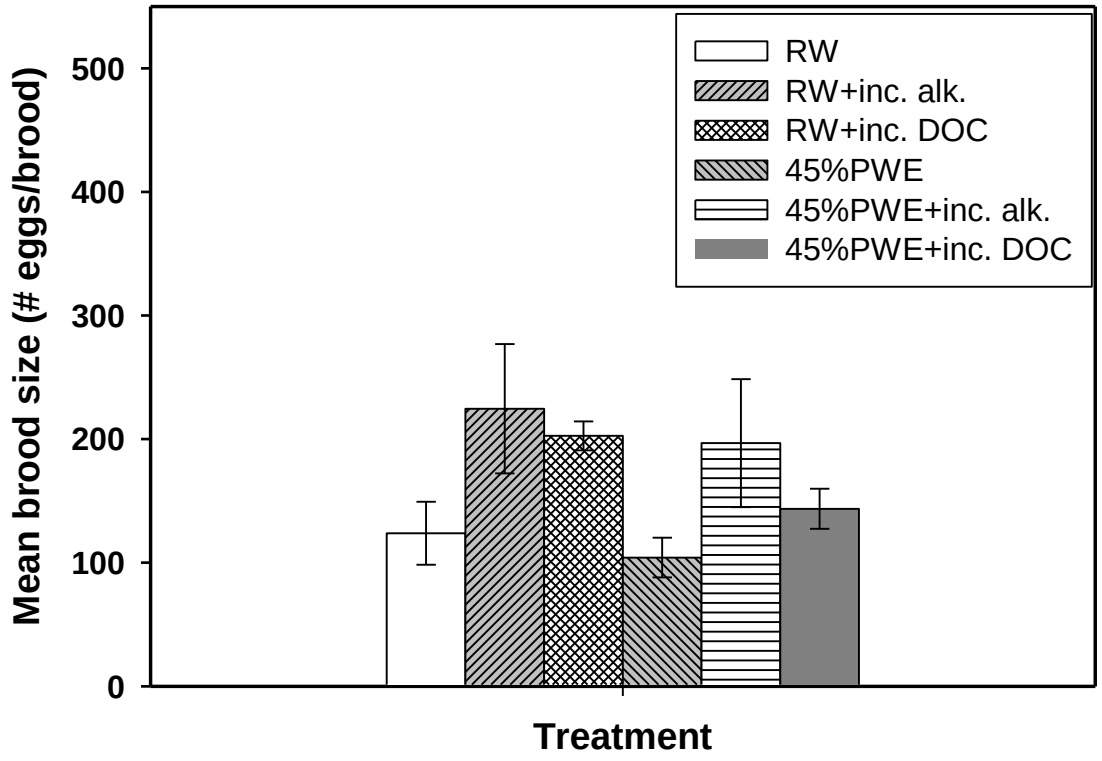

Figure 4-4 – Mean brood size (±S.E.) of fathead minnow (*P. promelas*) breeding pairs from unmodified and modified (increased alkalinity and increased DOC) reference water (RW) and 45% process water effluent (PWE) treatments. The unmodified treatments [reference water (RW) and 45% process water effluent (PWE)] had smaller mean brood sizes (# of eggs/brood), however there were no statistically significant differences among the treatments (Kruskal-Wallis; p=0.102).

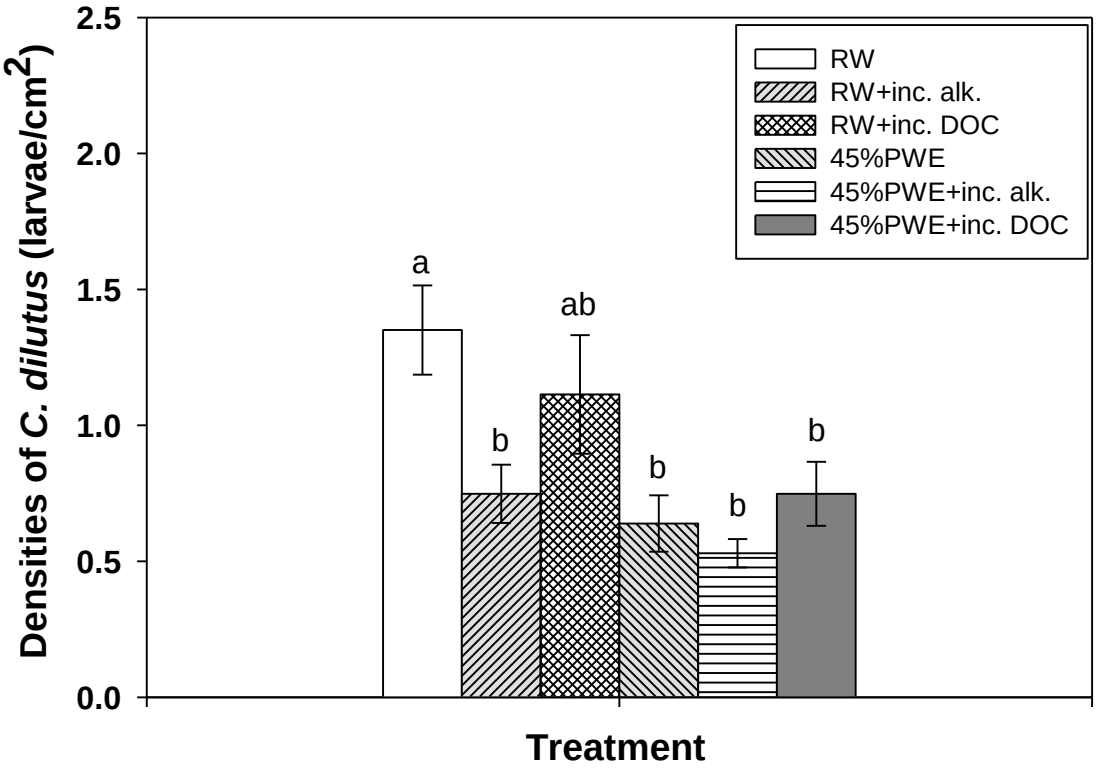

Figure 4-5 – Mean densities of *C. dilutus* larvae (±S.E.) sampled from unmodified and modified (increased alkalinity and increased DOC) reference water (RW) and 45% process water effluent (PWE) treatments on day 21 of the exposure period. Means that do not share letters are statistically different from one another (one-way ANOVA; Tukey HSD post hoc test, $p<0.05$).

the desired densities for satiation (1.48 larvae/cm^2, equivalent to 1 g/pair/day), and the densities were not significantly different between these two treatments (Tukey's HSD post hoc test; p=0.823). The RW+increased alkalinity treatment contained significantly fewer *C. dilutus* larvae than the unmodified RW treatment (-0.60 larvae/cm^2) (Tukey's HSD post hoc test; p=0.044). The unmodified 45% PWE treatment contained significantly fewer larvae than the unmodified RW treatment (-0.71 larvae/cm^2) (Tukey's HSD post hoc test; p=0.011). Larval densities in the 45% PWE+increased alkalinity and 45% PWE+increased DOC treatments were not significantly different from the unmodified 45% PWE treatment (Tukey's HSD post hoc test; p=0.993).

4.4 DISCUSSION

4.4.1 Reproductive impacts

The findings of our study suggest that elevated alkalinity (achieved by adding $NaHCO_3$) or DOC (achieved by adding commercial humic acid) can improve fathead minnow fecundity during chronic exposure to 45% PWE. Fish that were exposed to unmodified 45% PWE produced significantly fewer eggs over the 21-day exposure period relative to fish in the unmodified RW treatment - a typical response recorded in several previous studies with 45% PWE (Ouellet et al. 2013a, 2013b, Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a, 2011b). Fish fecundity improved markedly once 45% PWE treatments were modified to increase the alkalinity or DOC level in the exposure water. However, fish fecundity in both of the modified 45% PWE treatments was significantly less than the corresponding modified RW treatments (i.e., 45% PWE+increased alkalinity *vs.* RW+increased alkalinity, and 45% PWE+increased DOC *vs.* RW+increased DOC). This might be due to the additional energetic cost of metal handling and metabolism in 45% PWE treatments fish, since tissue-specific metal burden was

significantly greater in these fish relative to RW treatments fish, irrespective of water chemistry modifications (discussed below).

It appears that sodium bicarbonate or humic acid addition improves egg production in fathead minnows in general, as opposed to only ameliorating the effects of 45% PWE. In agreement with our observation, Mager et al. (2010) recorded improved egg production in fathead minnows when bicarbonate (pH 8.3) was added in the exposure water. Interestingly however, they reported that this effect was present only when bicarbonate was added into the control water but not in the treatment with metal (lead). Mager et al. (2010) also observed moderate improvements to fish fecundity when humic acid was added (4 mg/L) to the exposure water, with or without lead. In addition, greater egg production in fathead minnows has been reported at pH 7.5 compared to lower pH values (e.g., small decrease in eggs at pH 6.6 and dramatic decrease below pH 5.9) in the exposure water (Mount 1973). Although pH likely had a minor influence on fish egg production in our study, since the decrease in pH in the 45% PWE treatment relative to the RW treatment was relatively modest (~0.5 unit). Furthermore, pH in the 45% PWE treatment (~7.2) was within the acceptable range for the fish reproductive bioassay employed in this study (Ankley et al. 2001). Overall, these observations suggest that water chemistry itself may be an important factor in influencing reproductive capacity in fish.

At present, it is difficult to explain the exact reasons for improvements in fish fecundity due to changes in water chemistry. Mager et al. (2010) suggested that humic acid, because of its ability to bind biological surfaces, might contribute to better egg attachment on the breeding tiles. Sodium bicarbonate could perhaps improve the osmotic balance between the treatment waters and eggs, resulting in improved egg hardness and attachment to the breeding tiles, as well. Improvements in egg attachment to the breeding tiles would likely be indicated by greater mean

brood sizes from fish in water chemistry modified treatments, which was observed in the present study. It is also possible that greater sodium concentration in elevated alkalinity treatments might have ameliorated the energetic cost of osmoregulation, allowing fish to allocate more energy towards reproduction, which eventually led to improved fecundity. In the present study, the addition of sodium bicarbonate in alkalinity treatments increased the sodium concentrations from approximately 10 to 32 mg/L in the RW and from approximately 65 to 113 mg/L in the 45% PWE. It has been recently reported that egg production in fathead minnows generally improves with increasing sodium level in the water, with a pronounced effect at ≥25 mg/L (Squires 2011, Squires et al. 2013), which is comparable to the sodium level in our RW+increased alkalinity treatment. However, the 45% PWE treatments in the present study already contained high sodium levels in the water (>60 mg/L), which suggests that sodium may not be the only factor that influenced egg production in these fish.

In contrast, the addition of humic acid (as a sodium salt) in the RW and 45% PWE treatments resulted in a small and insignificant increase in dissolved sodium levels compared to that in the respective unmodified treatments, nevertheless resulted in increased egg production in fish, as well. This might have occurred due to the direct physiological effects of humic acid since it has been found to stimulate branchial sodium uptake and reduce paracellular permeability of the gill in fish (Matsuo et al. 2004, Wood et al. 2003). Both of these effects are expected to alleviate the osmoregulatory stress that freshwater fish experience due to the continuous loss of essential ions from the body through simple diffusion, and thus may facilitate egg production by fish. In addition, these effects may also ameliorate the disruption of essential ion homeostasis in fish induced by exposure to metals (e.g., copper) (Wood et al. 2011). It is therefore reasonable to suggest that humic acid influenced egg production in fathead minnows

exposed to RW or 45% PWE possibly because of its beneficial physiological effects, rather than by reducing metal bioavailability through increased complexation of dissolved metals (discussed below). To the best of our knowledge, this is one of few studies to report that humic acid exposure can augment egg production in fish, and further investigations are required to understand the mechanistic underpinnings of this effect. It is also important to note here that sulphate levels were greater than 20-fold higher in the 45% PWE treatments relative to the RW treatments in the present study. Although the egg production in fathead minnows have been found to decrease with increasing dissolved sulphate level (Squires et al. 2013), it is unlikely to be the case in the present study since we have previously recorded no decrease in egg production when fish were exposed to single metals (copper, nickel or selenium) along with almost identical levels of dissolved sulphate, as recorded in our 45% PWE treatments (Ouellet et al. 2013a).

4.4.2 Metal bioaccumulation

In addition to the amelioration of reproductive impairment, one of the other hypotheses of the present study was that elevated alkalinity or DOC in the 45% PWE treatment would reduce waterborne metal bioavailability and tissue-specific metal accumulation in fathead minnows due to increased complexation of free metal ions. Chronic exposure to 45% PWE resulted in increased accumulation of multiple metals in different tissues of fathead minnows, however, contrary to our hypothesis, we did not observe any protective effect of increased alkalinity or DOC on the metal(s) accumulation in any fish tissues. One of the possible reasons for the lack of protective effect might be that the increase in alkalinity or DOC produced a modest decrease in the free ion concentrations of most metals present in the 45% PWE exposure. For example, the four-fold increase in alkalinity in the 45% PWE treatment only produced 7-20% decrease in the free ion concentration of the major metals with the exception of copper (>90% decrease).

Similarly, the addition of humic acid (4 mg/L) into the 45% PWE exposure did not affect the free ion activity of most metals except cobalt and copper (7 and 70% decrease, respectively). This likely occurred because of two factors: (i) the actual increase of DOC in the exposure was marginal (only by 25%), and (ii) the complexation effect of DOC on metal speciation is metal-specific and depends on the relative affinity of binding of free metal ion(s) to DOC, and copper binds more strongly to DOC compared to any other metals present in 45% PWE [see Niyogi and Wood (2004) for review]. Perhaps, an unusually high sulphate level in the 45% PWE also influenced the relative complexation of different metals by bicarbonate or humic acid. Moreover, it is important to note that the geochemical speciation models like MINTEQ, which was used in the present study for the estimations of free metal ion activities, are based on short-term metal-ligand binding under equilibrium condition, and whether such equilibrium assumptions are valid under prolonged exposure remains an open question.

Although the protective effects of DOC and/or high pH/alkalinity on metal accumulation and toxicity to fish during acute exposures to metal(s) or metal-mixtures are well-documented (Burnison et al. 2006, Klinck et al. 2005, Komjarova and Blust 2009, Niyogi et al. 2008, Pyle et al. 2002, Richards et al. 2001), recent evidence indicate that these water chemistry parameters often provide much less or no protection during chronic metal exposures. Mager et al. (2010) observed no significant effect of increased alkalinity (added as 500 μM $NaHCO_3$) on lead accumulation in fathead minnows during chronic waterborne exposure to lead, despite a ~30% decrease in free lead ion concentration in the exposure water. Interestingly though, they reported amelioration of lead accumulation and toxicity in fish when DOC level was elevated in the exposure (added as 4 mg/L humic acid), which also resulted in a ~30% decrease in free lead ion level. On the other hand, Brauner and Wood (2002a) reported that increased DOC (added as 12

mg/L humic acid) decreased mortality but did not ameliorate the adverse physiological response in rainbow trout chronically exposed to waterborne silver. Currently, very little is known about the effects of alkalinity or DOC on the bioavailability and toxicity of metals to fish during chronic exposures to metal mixtures. Recently, Kamunde and MacPhail (2011) examined the effect of increased DOC (added as 5 mg/L humic acid) during chronic exposure to a waterborne mixture of copper, cadmium and zinc to rainbow trout. They reported that the protective effect of DOC against metal(s) accumulation was variable (absent or partial/complete) depending on tissue and exposure duration. More importantly, they also concluded that the modeled free metal ion activities in the exposure could only explain the cadmium but not copper and zinc accumulation in fish. Overall, our findings on the effects of increased alkalinity and DOC on metal(s) accumulation in fish during chronic exposure to complex metal-mixture are more or less consistent with previous observations, and provide further evidence that the free ion activity alone is not a reliable predictor of chronic metal accumulation or toxicity in fish.

4.4.3 Biological endpoints

Apart from cumulative egg production and tissue-specific metal accumulation, none of the other biological endpoints examined in the present study were influenced by 45% PWE, as well as increased alkalinity or DOC. Fathead minnows exposed to 45% PWE typically do not show changes to biological endpoints such as GSI, LSI, or condition factor (Ouellet et al. 2013a, Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a). The effects on hatch rate, larval deformities, and egg size have been variable in fish chronically exposed to 45% PWE. Rickwood et al. (2006a) observed a decrease in hatching success and an increase in larval deformities when fathead minnows were exposed to 45% PWE simultaneously via water and diet, however no effects when fathead minnows were exposed via water only. In subsequent

studies, Rozon-Ramilo et al. (2011a) and Ouellet et al. (2013a, 2013b), however, did not observe any significant differences in hatching success or larval deformities during chronic multi-trophic exposure to 45% PWE. In addition, a decrease in egg size was previously recorded in fish during chronic exposure to 45% PWE, although the effect was not always consistent across similar experiments (Ouellet et al. 2013a). It is possible that egg sizes might have been affected by 45% PWE, instead of decreases in egg production, in some experiments. Consequently, fathead minnows produced a similar number of eggs but in smaller sizes, instead of producing fewer eggs. Regardless, the egg size was not affected by any of the treatments carried out in the present study.

4.4.4 The role of diet and Chironomus dilutus

The presence of live *C. dilutus* larvae as a food source in multi-trophic MME exposures have been shown to be an important factor that contributes to metal mixture accumulation and toxic responses in fish (Rickwood et al. 2006a, Rozon-Ramilo et al. 2011b). The present study indicates that the metal bioavailability via diet during chronic 45% PWE exposure was not altered by increased alkalinity or DOC, since *C. dilutus* metal burden was generally similar across all three 45% PWE treatments. However, we recorded differences in densities of *C. dilutus* larvae among treatments, which were measured at the end of the exposure period. A decrease in densities of *C. dilutus* larvae due to 45% PWE exposure have also been reported in previous studies (Hruska and Dubé 2004, Rozon-Ramilo et al. 2011a). This reduction in food source has been implicated as a factor contributing to reduced egg production in fathead minnows (Ouellet et al. 2013b). In the present study, egg production in fish might also have been affected by the decrease in food availability in the unmodified 45% PWE treatment. However, a difference in food availability alone does not explain the decrease in fish fecundity

induced by 45% PWE, since fish in both of the modified 45% PWE treatments produced as many eggs as the fish in unmodified RW treatment, despite encountering a similar decrease in *C. dilutus* density. This further emphasizes the point that sodium bicarbonate and humic acid probably facilitated fish egg production by directly influencing the physiology of fish.

4.4.5 Conclusions

Overall, the findings of the present study corroborate that environmentally relevant chronic exposure to MME can lead to significant tissue-specific accumulation of metals and reduce fish fecundity. Modifications of the water chemistry through the addition of sodium bicarbonate (increased alkalinity) or humic acid (increased DOC) restored or improved the reproductive output, but did not alter the tissue-specific metal accumulation profile, in fish exposed to MME. Interestingly, the addition of sodium bicarbonate and humic acid also resulted in significantly greater fecundity in fish exposed to reference water. Fish in the MME exposures, irrespective of water chemistry modifications, encountered reduced food availability (*C. dilutus* density). Collectively, these results indicate that reproductive impairment in fish induced by chronic MME exposure was not solely mediated by the accumulation of metals and indirect effects such as reduced food availability probably contributed to the effect. However, it appears that sodium bicarbonate or humic acid addition enabled fish to compensate adverse reproductive consequences of MME exposure, possibly through their direct beneficial physiological effects. Further studies are required to examine the potential usefulness of water chemistry modifications in reducing the toxicity of MME to biota in the receiving environment.

5 CHAPTER 5 - GENERAL DISCUSSION

5.1 SUMMARY

Metal mining activities can negatively affect water quality, and subsequently, aquatic life that is dependent on clean water for survival and reproduction. Evaluating effects of anthropogenically released metals from mining activity on the aquatic environment is a necessary step in determining whether those contaminants are harmful. Many studies, for example, have examined mining effluents to determine whether or not they contribute to effects on benthic invertebrates or fish in the aquatic environment (see section 1.1). However, in order to improve treatment options for mining operations, understanding sources of effects, importance of exposure routes in causing effects, and possible methods for mitigating effects must first be examined. Therefore, the objectives of the research included in this thesis were to examine several possible sources of effects, both direct (e.g. causative metals – Chapter 2) and indirect (abundance of food – Chapter 3), as well as to evaluate whether water chemistry modifications (increased alkalinity and NOM) could be used as a method of mitigating toxic effects of mining effluents to fish (Chapter 4). The specific objectives and major findings of each chapter are presented below in a flowchart (see Figure 5-1). Although the approach taken in this thesis is specific to a particular effluent, 45% PWE from a Canadian metal mine, the methodology and findings can be applicable to other effluents.

In general, findings described in Chapter 2 (Ouellet et al. 2013a) suggest that copper, nickel, and selenium, alone or in a mixture, likely were not contributing to reduced fecundity in fathead minnows exposed to the 45% PWE, under the metal concentrations and water chemistry conditions examined (i.e. ~60 µg/L Cu, ~90 Ni µg/L, and ~10 Se µg/L, pH 6.9-7.6 and hardness ~575-700 mg/L as $CaCO_3$). Interestingly, the concentrations of copper and selenium in the 45%

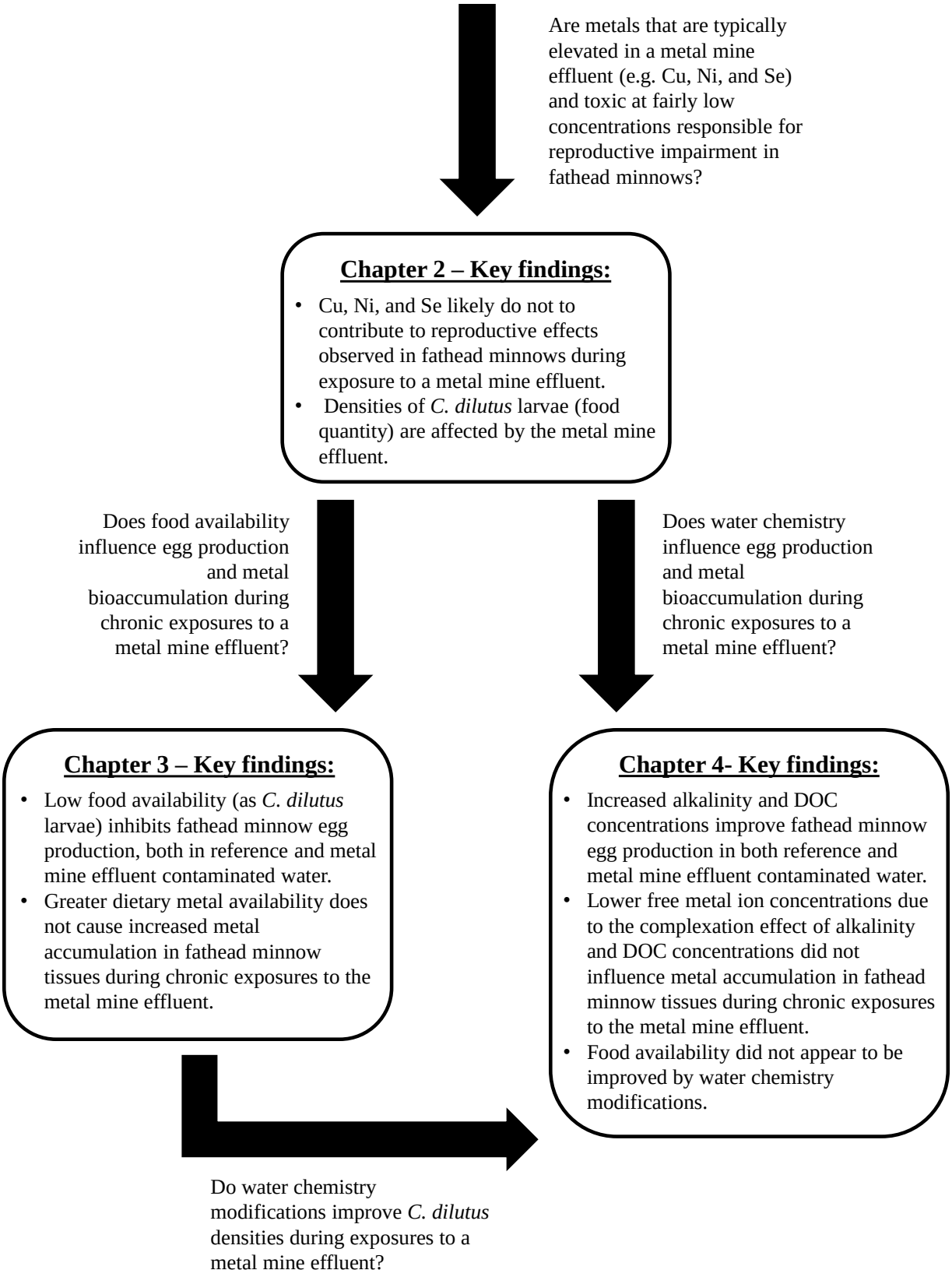

Figure 5-1 – Schematic diagram of the major findings of each chapter from the present thesis.

PWE were significantly greater than Canadian Water Quality Guidelines (4 µg/L at hardness of ≥180 mg/L as $CaCO_3$ and 1.0 µg/L, respectively), but below the recommended guidelines for Ni (150 µg/L at hardness of ≥180 mg/L as $CaCO_3$) (CCME 2008). Although copper, nickel, and selenium were at elevated concentrations in the MME, the modest accumulation of these metals in fathead minnow tissues suggests that they are not readily bioavailable to the fish either via water or diet. In general, significant accumulation of metals was recorded in *C. dilutus* larvae, suggesting that dietary exposure was probably the predominant route of metal accumulation in fish. Importantly though, no link between metal bioaccumulation patterns and reduced fecundity in fathead minnows was found under the test conditions.

The results suggest that copper, nickel, and selenium were not important factors in impacting fecundity of fathead minnows exposed to the MME at the concentrations that were examined. Nevertheless, the interactions among various metals can strongly influence toxic responses in aquatic organisms (Norwood et al. 2003), and thus cannot be completely ignored. It is possible that the interactions among all of the metals that were present in the MME at elevated concentrations, including copper, nickel, and selenium, were partly responsible for inducing reproductive impairment in fathead minnows exposed to 45% PWE. In addition, another potentially important factor, the differences in food availability, contributing to the reproductive toxicity of 45% PWE were also identified in Chapter 2. Specifically, densities of *C. dilutus* in 45% PWE streams were generally less than those in the metal or reference treatments, although not always statistically less (Ouellet et al. 2013a, Rickwood et al. 2006a, Rozon-Ramilo et al. 2011a). Based on this observation, it was hypothesized that differences in food availability could be responsible for lower fecundity in fathead minnow breeding pairs, as altered prey abundance can indirectly affect the health of fish (Campbell et al. 2003, Rasmussen et al. 2008).

Subsequently, the role of food availability in influencing fathead minnow reproduction and metal bioaccumulation in an MME was examined in Chapter 3 (Ouellet et al. 2013b).

There were two central themes to the research performed in Chapter 3. The first theme of Chapter 3 was to examine whether food quantity could influence fecundity of fathead minnows, while the second theme was to determine whether food quantity could influence metal accumulation in target tissues of the fish (e.g., liver, gonads, gills, and carcass) during chronic exposure to 45% PWE. Although dietary intake of metals is known to be an important source of metal uptake and toxicity in fish (Niyogi and Wood 2003), the role of food quantity (*C. dilutus* density) in influencing toxic responses from complex metal-mining effluent exposures has not been examined previously. The findings from Chapter 3 confirmed that food quantity was an important factor in influencing fecundity of fathead minnows, and interestingly its effect was found to be greater than the effect caused by direct exposure to 45% PWE (Ouellet et al. 2013b). These findings are particularly important as they suggest that indirect effects from decreased food availability (e.g., decreased macro-invertebrate densities) in a contaminated environment could be detrimental to the reproductive performance in fish, even if the contaminants themselves are not directly toxic to the fish. It is to be noted though that the laboratory study performed in Chapter 3 included only a single food source (*C. dilutus* larvae), therefore it is difficult to predict the consequences of reduced food availability in an environment where other food sources are likely to be present.

In addition to the importance of food quantity in influencing fathead minnow reproduction, results from Chapter 3 also indicated that metal concentrations in tissues were largely dependent on food availability, not metal exposure, which highlights the importance of considering growth dilution and food consumption in metal exposure and bioaccumulation studies. Furthermore,

studies that have found changes in GSI and condition factor due to metal exposures have often encountered difficulty determining whether these changes occurred due to direct or indirect effects (discussed in Rasmussen et al. 2008). In case of MMEs, it is probable that food availability is critically important factor, particularly when examining GSI and condition factor changes in fish.

Lastly, the research in Chapter 4 was performed in order to evaluate whether water chemistry influences reproductive responses of fathead minnows exposed to the MME, and whether water chemistry modifications could be used as a method to improve effluent quality and mitigate its toxic implications. Key findings presented in Chapter 4 suggest that increased alkalinity (supplemented as sodium bicarbonate) and NOM (supplemented as commercially available humic acid) significantly improved reproductive performance of fathead minnows during exposure to 45% PWE. However, neither of the two water chemistry variables examined produced any effects on the accumulation of metals in fathead minnow tissues, suggesting that these variables are probably not nearly as effective in influencing metal bioavailability as observed during acute exposures and/or single metal exposures, at least under the modest (and environmentally achievable) modifications that were performed. It is evident that further research is needed to understand the influence of various water chemistry parameters on the bioavailability of metals to aquatic organisms during chronic metal-mixture exposure scenarios. Overall, the metal bioaccumulation results in fathead minnow tissues presented in Chapter 4 were not surprising, since the results from Chapters 2 and 3 strongly indicated that exposure to metals were not directly responsible for reduced fecundity in fathead minnows during exposure to 45% PWE. It is however important to note that sodium bicarbonate and humic acid supplementation did not entirely mitigate the effects of 45% PWE, since the egg production in

fathead minnows exposed to 45% PWE under modified water chemistry was always lower relative to that in the comparable reference modified water chemistry treatments (alkalinity or NOM).

Interestingly, although food quantity was found to influence fathead minnow egg production in Chapter 3, it did not appear to influence egg production under the water chemistry modifications performed in Chapter 4. We found that egg production increased both in the reference water and MME treatments along with the increases in alkalinity and DOC, and this occurred despite no significant alterations in *C. dilutus* densities. This suggests that under the ambient condition 45% PWE reduces food availability, which eventually decreases the fecundity of fathead minnows. On the other hand, alkalinity and DOC modifications appear to improve egg production in fathead minnows regardless of the food availability.

5.2 RECOMMENDATIONS FOR FURTHER RESEARCH

The research performed in this thesis addressed several important issues that are relevant to the environmental impacts of metal mining operations, namely exploring causative and modifying factors of chronic MME toxicity to a model fish species, *P. promelas*. Nevertheless, several issues still exist as discussed below, which will require further exploration.

5.2.1 Causal factors

Although the research performed in this thesis examined several possible causative factors of MME toxicity, we were unable to determine which factor(s) of the MME was directly contributing to responses in fish. Specifically, does the metal bioaccumulation have any role in inducing toxic effects in biota (e.g., reduced densities of *C. dilutus* and reduced fathead minnow egg production)? Food quantity appears to have contributed to reduced fecundity in fathead minnows under ambient condition, however this research did not examine causes of decreased *C.*

dilutus densities, beyond examining metal accumulation in tissues. Copper, nickel, and selenium were not found to cause reduced densities of *C. dilutus* larvae, although they did accumulate in the tissues. Rubidium and thallium also accumulated in *C. dilutus* larvae (Chapter 3), however thallium was not elevated in the other studies performed in this thesis (Chapter 2 and Chapter 4) and therefore appears to have minimal potential to cause effects in the MME. The potential for rubidium to contribute to toxic effects in aquatic organisms has been discussed quite thoroughly by Rozon-Ramilo (2011b). Few studies have examined the toxicity of rubidium, however rubidium appears to have the potential to biomagnify through the food chain (Campbell et al. 2005) and impair spermatogenesis in fish (Yamaguchi et al. 2007), thus contributing to reproductive impairment in fish. Because so few studies have examined the toxicity of rubidium, it would be worthwhile to explore its potential to cause reproductive impairment in fish during chronic exposure to an MME. Additional compounds that are added to MMEs during the treatment process, such as lime or organic flocculants, might also have the potential to adversely affect *C. dilutus* as well as fish during MME exposures, and would be worth examining. These chemical agents have not previously been examined in laboratory mesocosm studies because little information is available to indicate which specific chemicals are added in the treatment process. Because water chemistry changes did not altogether eliminate the toxic effects of MME (Chapter 4), it is quite probable that chemical agents other than metals are responsible for the observed effects in MME.

5.2.2 Food availability

The mesocosm system used in the studies performed in this thesis was developed to increase environmental realism by including a live food source, however it is still a simplified version of a real ecosystem. Fish in the environment are likely to have multiple sources of food.

Fathead minnows, for example, will feed on various crustacean, cladoceran, or copepod species in the wild (Held and Peterka 1974). It would be interesting to investigate whether or not MMEs contribute to reduced densities of other prey species that fathead minnows would consume. On the same note, it would also be valuable to develop multi-trophic mesocosms with several food sources in order to evaluate whether fathead minnows would undergo reduced fecundity during chronic exposure to MME if multiple food sources were available. Interestingly, during the water chemistry modification study (Chapter 4), densities of *C. dilutus* larvae were lower in modified-alkalinity treatments (both reference water and MME), yet improved egg production in fathead minnows was recorded. It would also be useful to evaluate the ameliorating effect of alkalinity under a longer chronic exposure period, specifically to determine whether fish are able to sustain improved reproductive performance while being subjected to prolonged low food ration.

5.2.3 Water chemistry modifications

Results presented in Chapter 4 suggest that alkalinity and DOC modifications could potentially be a useful treatment option for mitigating the toxic effects of MMEs to the resident biota. However, the long-term environmental implications of these types of modifications should first be examined in order to ensure their safe application. Moreover, regulators would need to decide whether egg production improvements in fish would be a worthwhile endpoint to consider, since food availability might remain lower in the aquatic environments receiving the metal mine effluents. It is also not apparent whether such water chemistry modifications would ultimately ameliorate metal accumulation in fish – a key assessment endpoint in the Canadian EEM program.

5.2.4 Additional endpoints

The research performed as part of this thesis examined many different endpoints in fathead minnows (e.g., fish morphometrics, metal bioaccumulation, fish fecundity, and larval deformities) during multi-trophic exposures to an MME. The present research, as well as other EEM studies, provides strong evidence that inhibitory effects (e.g., decreased morphometrics, reproduction, species diversity and abundance) are the most-common and sensitive responses observed in aquatic systems exposed to MMEs (Environment Canada 2012, Ouellet et al. 2013a, 2013b). As a result, it is probable that altered food-web dynamics and energetic changes might be a primary cause of many of the commonly observed inhibitory effects in resident fish populations. Alterations in food webs and fish energetics have been observed in metal contaminated lakes in Canada (Iles and Rasmussen 2005), and therefore, including biochemical endpoints for assessing the energetic status of fish (e.g., glycolytic enzymes such as lactate dehydrogenase) in laboratory or on-site multi-trophic mesocosm studies could be useful. This may help in characterizing the cause and effect relationship in fish exposed to MME, since monitoring of feeding behaviour of fish in mesocosms is quite difficult.

5.2.5 Recommended improvements to the multi-trophic mesocosms

Although the multi-trophic mesocosms that were used in the present thesis are an accepted component of EEM, several improvements could be made to the system in order to reduce variability, the potential of pseudoreplication, and fungal infections in fish eggs, and ultimately improve their usefulness in environmental monitoring research. Specifically, the maximum sample size achievable in the mesocosms used in Chapters 2, 3, and 4 was eight replicates per treatment. Fathead minnow egg production is often quite variable, and therefore a minimum of 10 replicates per treatment would improve the power to detect significant differences in egg

production. As well, the mesocosm system that was used in the present thesis employs a water-recirculation manifold, thus pseudoreplication could be a concern if any replicate were to become contaminated during an experiment. Replacing the recirculating pumps with peristaltic pumps would eliminate the possibility of pseudoreplication. Lastly, fungal infections in fish eggs were quite common in each of the studies performed in the present thesis (see Appendix Figure A-2). The egg cups that were used for monitoring hatch rate and determining larval deformities tended not to provide sufficient air flow and agitation to prevent fungal infections from spreading throughout the eggs. Consequently, these egg cups should be redesigned with the goal of providing a maximum number of air bubbles and better flow of treatment water within the egg cups. Also, once infected eggs occurred, the eggs cups appeared more susceptible to future fungal infections, therefore it is recommended that infected egg cups should be disposed of and replaced after each use.

5.3 CONCLUSIONS

The approach taken in the present thesis highlights some of the difficulties in identifying causal factors of adverse effects induced by complex metal mine effluent exposure in aquatic ecosystems. Although the effects were initially hypothesized to occur due to direct metal exposures through both the water and diet, it appears that the effects are likely more complex and at least partially induced by the altered food availability (indirect effects) and some additional uncharacterized aspects of the MME (e.g., cumulative effects of all the elevated metals, flocculants). Overall, the main objectives presented in Chapter 1 have been addressed, which would considerably enhance our understanding of the impacts of MMEs in the receiving aquatic ecosystems. Nevertheless, assessing the environmental impacts of complex industrial effluents is a challenging proposition that requires a step-by-step evidence-driven exploratory approach,

and thus the unresolved issues delineated in this thesis would be useful towards achieving that goal.

6 CHAPTER 6 - REFERENCES

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7 APPENDIX

7.1 LIST OF APPENDIX TABLES

7.2 LIST OF APPENDIX FIGURES

Table A-1 – Concentrations of anions and elements (±S.E.) measured from all unmodified 45% PWE treatments analyzed from all data chapters of the present thesis (Chapter 2 to Chapter 4).

	Br-	Cl-	F-	NO3-	NO2-	PO4 3-	SO2 4-
mean	0.35*	45.82	0.37*	0.80	1.29	3.63*	645.62
S.E.	0.22	8.63	0.21	0.39	0.38	2.15	30.93
units	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L

	Al	Sb	As	Ba	Be	Bi	B	Cd	Ce	Cs	Cr	Co	Cu	Eu	Ga	Fe
mean	10.79	1.64*	0.72*	19.58	0.31*	0.63*	44.87	0.17	0.63*	0.63*	0.64*	1.78	49.93	0.63*	0.63*	52.83
SE	1.46	0.80	0.13	3.23	0.06	0.13	8.62	0.05	0.13	0.13	0.15	0.74	16.07	0.13	0.13	17.11
units	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L

	La	Pb	Li	Mn	Hg	Mo	Ni	Nb	K	Rb	Sc	Se	Si	Ag	Na
mean	0.63*	0.63*	23.08	13.52	0.18*	1.05	90.00	0.63*	14.20	17.76	0.63*	7.14	436.8	0.18*	57.97
SE	0.13	0.13	7.77	4.74	0.11	0.25	19.16	0.13	72.68	5.85	0.13	1.40	87.61	0.11	13.05
units	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	mg/L	µg/L	µg/L	µg/L	µg/L	µg/L	mg/L

	Sr	S	Te	Tl	Th	Sn	Ti	W	U	V	Y	Zn	Zr
mean	445.58	223.83	0.75	0.19*	0.63*	4.97	1.01	0.63*	0.63*	0.63*	0.63*	17.89	1.38*
SE	92.84	327.76	0.25	0.10	0.13	2.53	0.20	0.13	0.13	0.13	0.13	1.12	0.72
units	µg/L	mg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L	µg/L

* indicates that at least two of the measurements for that element in 45% PWE were either at or below the detection limit. Therefore, the means of these values are likely presented at a concentration that is greater than what would be observed in 45% PWE.

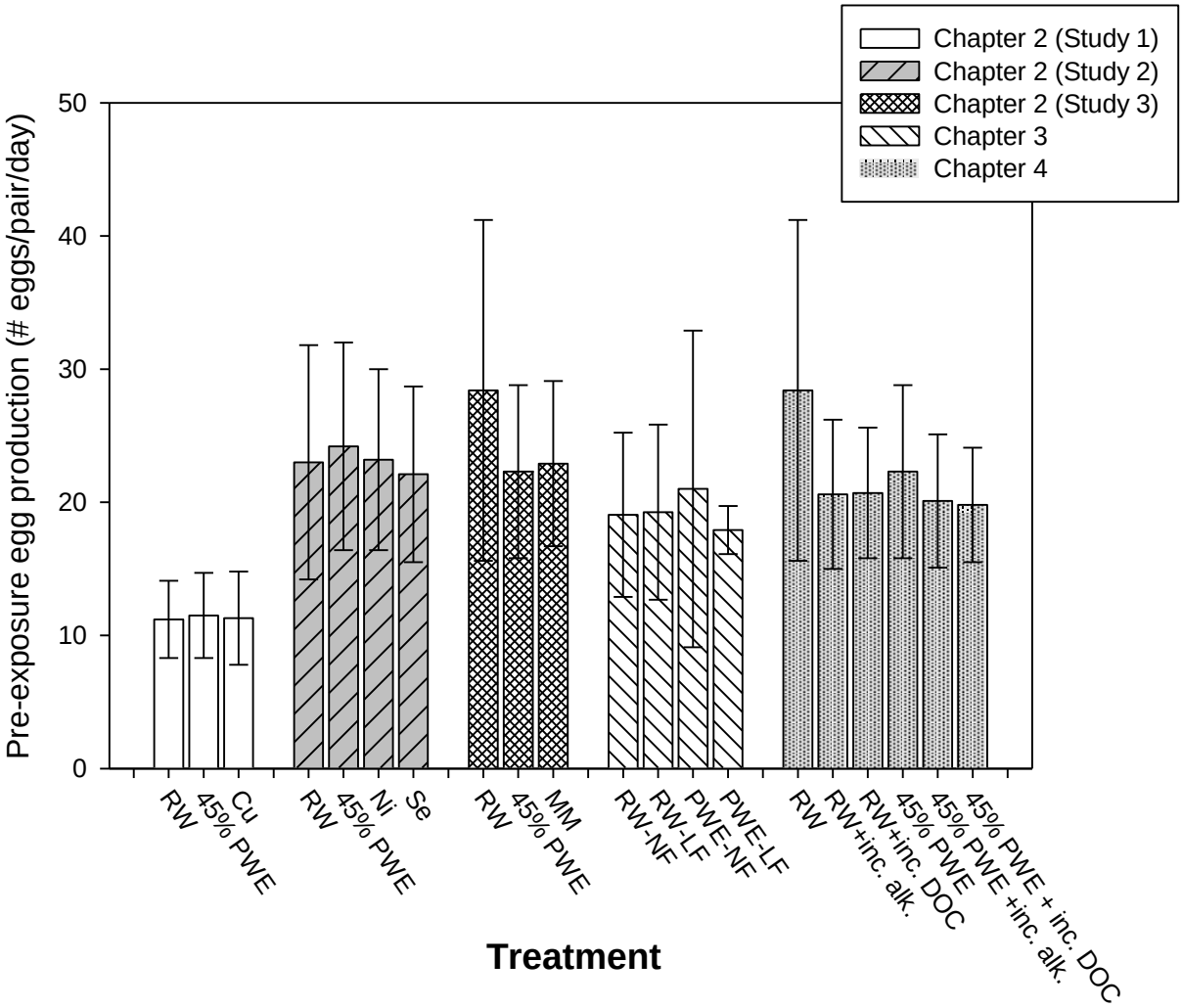

Figure A-1 – Mean egg production per fathead minnow (*P. promelas*) breeding pair (# of eggs/pair/day) over the pre-exposure period of each data chapter from the present thesis. Values are means ± S.E.

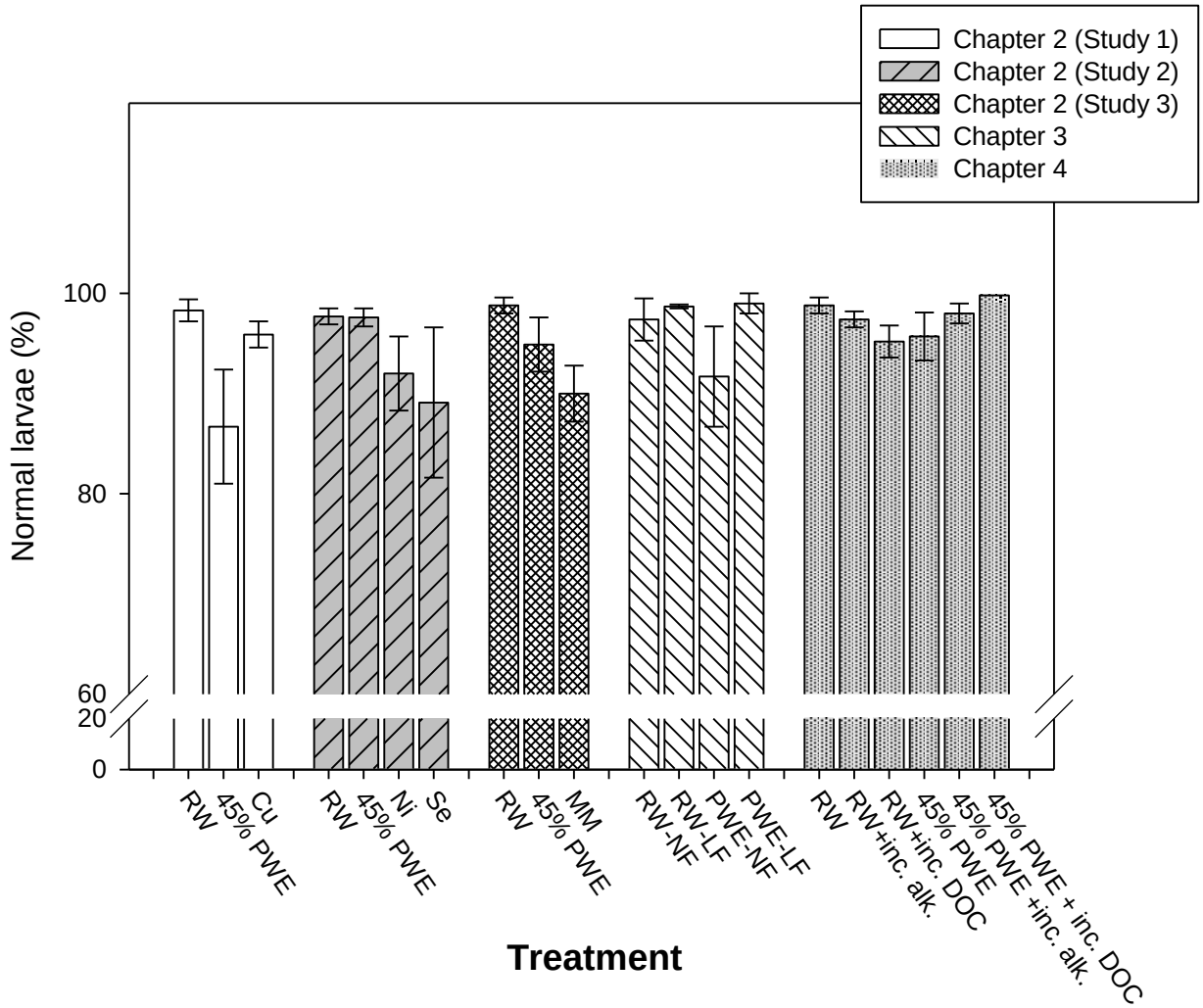

Figure A-2 – Mean percent of larvae at hatch without deformities per fathead minnow (*P. promelas*) breeding pair over the 21-day exposure period of each data chapter from the present thesis. Values are means ± S.E.

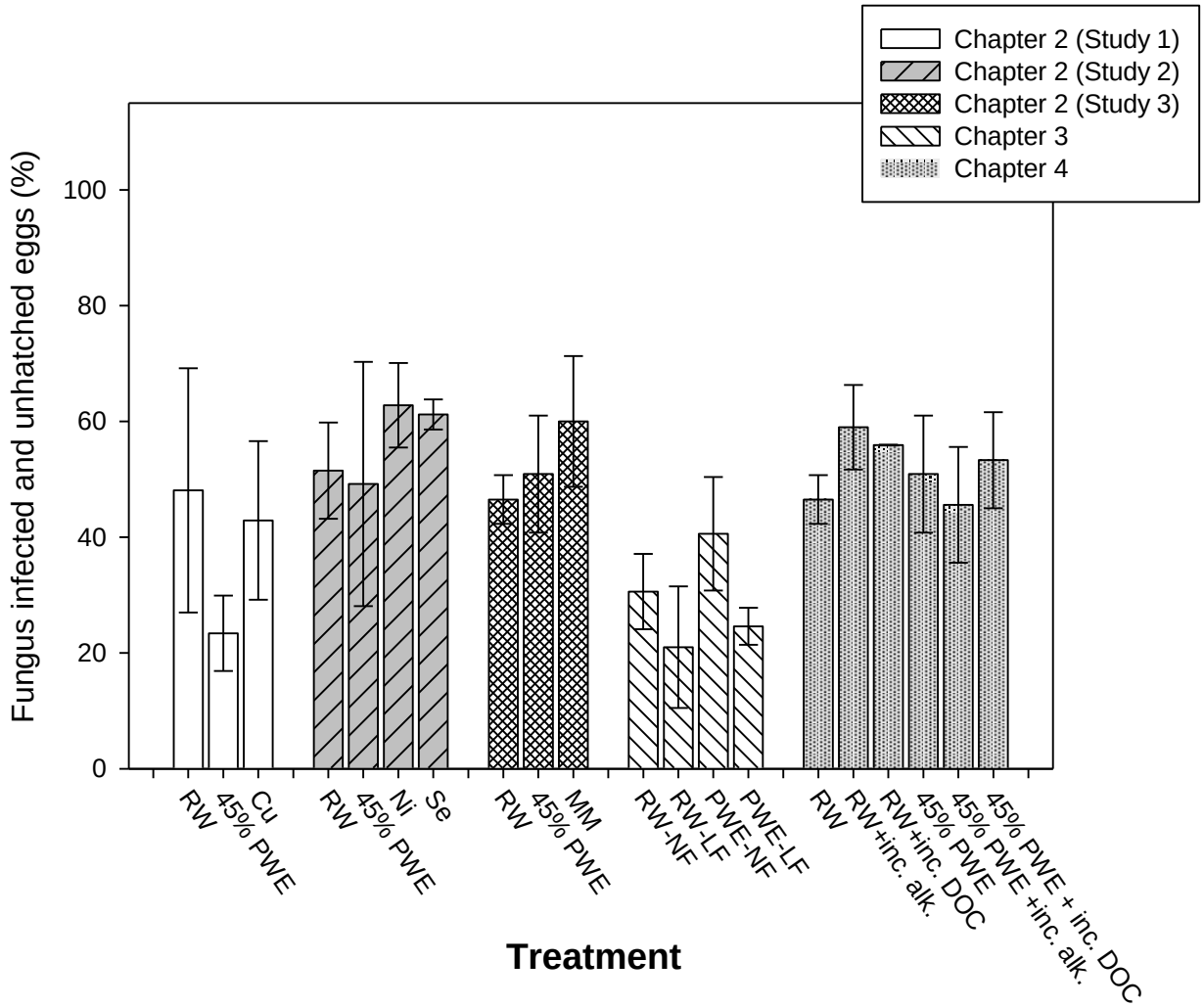

Figure A-3 – Mean percent of fungus infected and unhatched eggs per fathead minnow (*P. promelas*) breeding pair over the 21-day exposure period of each data chapter from the present thesis. Values are means ± S.E.

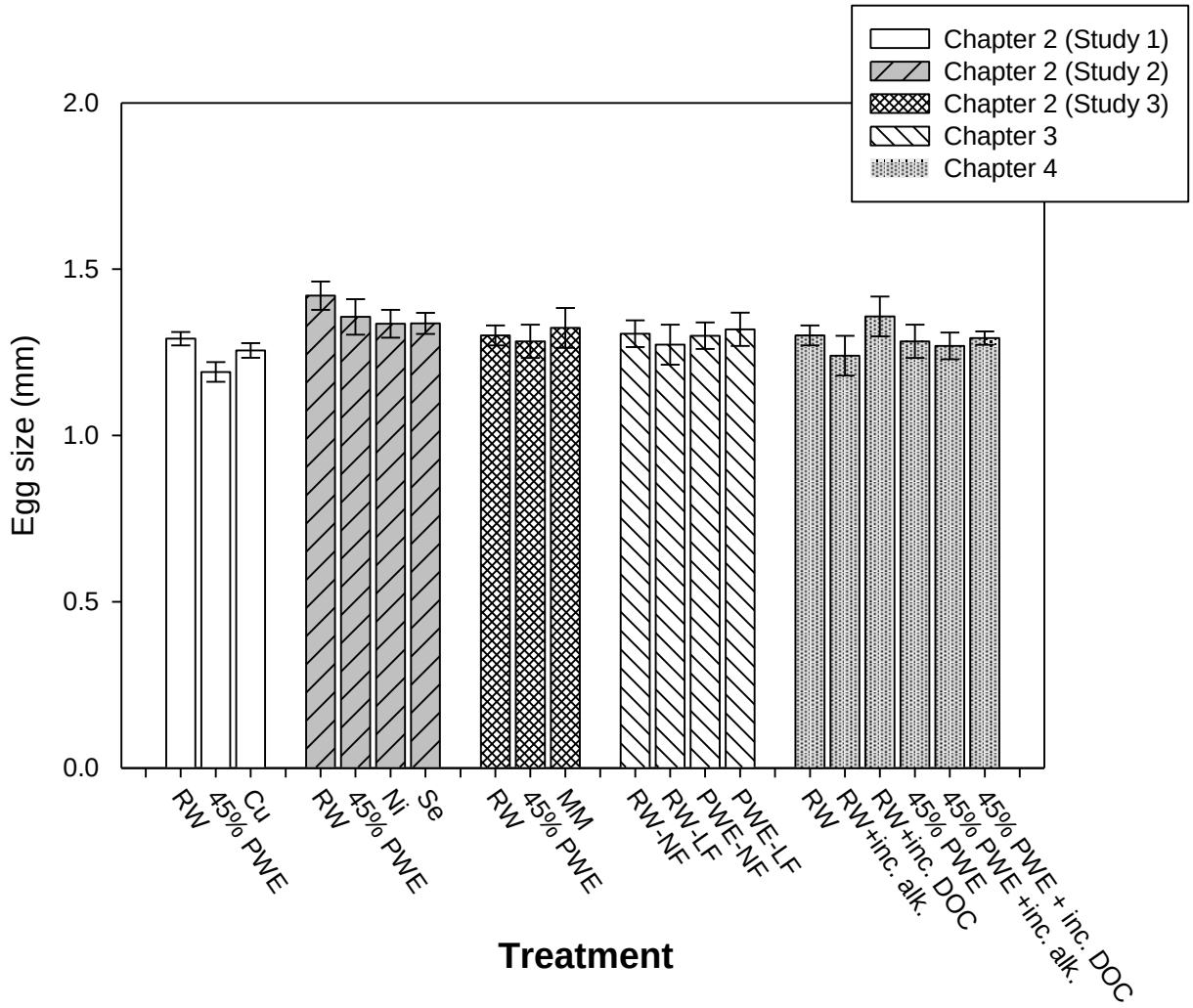

Figure A-4 – Mean egg size per fathead minnow (*P. promelas*) breeding pair (mm) over the 21-day exposure period of each data chapter from the present thesis. Values are means ± S.E.